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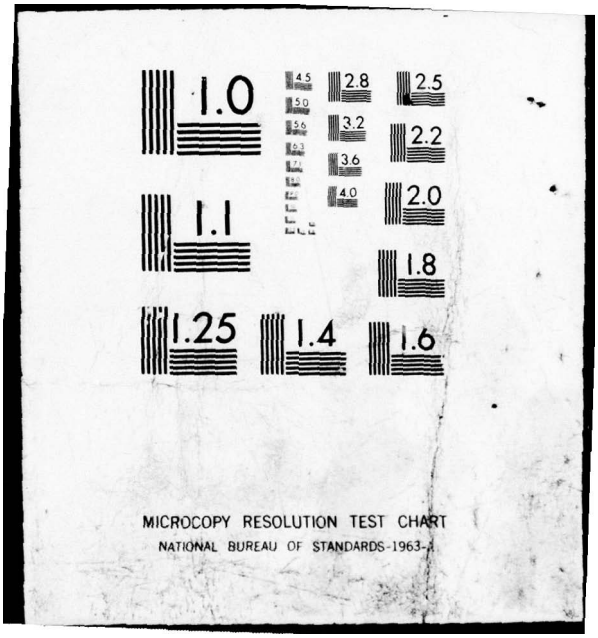
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An experimental model of shock and trauma was developed in isolated rabbit hearts subjected to low levels of coronary arterial flow (ischemia). A high level of glucose and insulin was beneficial to the heart subjected to ischemia; equiosmolar mannitol had no effect. A blood-perfused rabbit heart was developed using outdated human red blood cells. The performance data of the blood-perfused heart under well-oxygenated conditions is reported.

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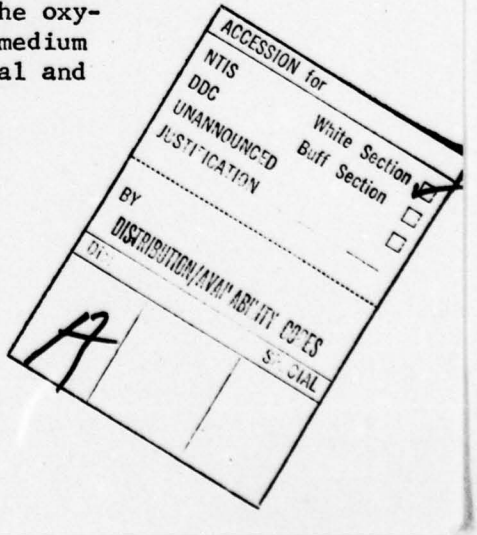
NR 207-014
Carl S. Apstein, M.D.

1. Summary

The overall goal of this project is to improve the treatment of trauma and shock. The specific approach is to utilize red blood cells which enhance oxygen delivery to tissues. The test system consists of an isolated rabbit heart which is subjected to global ischemia (reduction in coronary arterial blood flow); the isolated heart represents an experimental model of the trauma and shock state. During the first 15 months of this project we have developed and fully characterized the contractile, mechanical, metabolic, and morphologic properties of the isolated buffer-perfused rabbit heart preparation. These studies constitute a "control" or reference baseline to which studies in the blood-perfused rabbit heart can be compared. We have subsequently modified the buffer-perfused heart preparation so that the perfusate can be recycled. We have performed a large number of studies to define the ideal suspending medium for red blood cells and specifically have investigated the effect of different glucose concentrations and the presence of insulin. Groups of hearts were subjected to two different degrees of global ischemia, to model two different degrees of trauma and shock, and the effect of different concentrations of glucose $\pm$ insulin was studied. Our results (see below) clearly demonstrate the protective effects of a high blood glucose and insulin level on cardiac tissue subjected to ischemia (trauma and shock state). These studies also validated our methodology for measuring cardiac damage.

After having defined the characteristics of the buffer-perfused heart, its response to ischemia, and the effect of different substrate concentrations in the perfusion medium, we have begun a series of experiments perfusing isolated well-oxygenated rabbit hearts with red blood cell suspensions. Nine blood-perfused experiments have been performed as of this date (1 June 1978). In our most recent experiments, stable cardiac contractile and metabolic function has been achieved for greater than two hours. These studies, therefore, provide a good scientific foundation for proceeding with experiments in which the oxyhemoglobin state, or other hematologic or suspending medium components, will be altered and the effects upon normal and ischemic cardiac muscle observed.



2. Introduction

During the trauma and shock state, the arterial blood pressure is low and arterial perfusion of tissues, necessary to sustain life, is critically diminished. The reduction in tissue perfusion (ischemia) causes a decrease in tissue substrate delivery, a decrease in metabolic washout, and a decrease in oxygen delivery with resulting tissue hypoxia. This pathophysiologic combination results in progressive tissue dysfunction and ultimately cellular and organ necrosis and death. Because cardiac muscle has the highest oxygen demand of any organ in the body, and because continuous cardiac function is obviously necessary for life, the heart is particularly susceptible to the shock state and also the most important organ to preserve during shock; if cardiac pump function cannot be preserved during the shock state survival is not possible. Conversely, if cardiac pump function can be enhanced or preserved, then arterial perfusion of the entire body can be improved.

Accordingly, the overall goal of this research project was to develop an experimental model of the shock and trauma state as it affects the heart. This experimental system would then serve as a testing ground for new methods which could preserve or enhance cardiac function during the shock state. The first such method to be evaluated is the enhancement of oxygen delivery to tissues by alteration of the oxy-hemoglobin affinity state in circulating red blood cells. Other hematologic factors, or components of the red blood cell suspending medium could also be tested in this system to assess their effect on tissue subjected to the shock state.

Recent studies in the Cardiac Muscle Research Laboratory at the Boston University School of Medicine have shown that both normal and ischemic cardiac muscle function is very sensitive to small changes in tissue oxygen tension. Very small differences in oxygen delivery to the ischemic myocardium protect and preserve myocardial integrity so that contractile function returns to normal with subsequent reperfusion, i.e. in the post-shock state (Apstein, C.S. et al: Graded Global Ischemia and Reperfusion; Cardiac Function and Lactate Metabolism. Circulation 55, 864-872, 1977). Thus the response of the ischemic myocardium in our isolated heart test system can serve as a very sensitive measure for agents which have the potential to enhance tissue oxygen delivery or preserve cardiac function in the shock state by some other mechanism. Contractile function in the normal heart is also critically sensitive to oxygen delivery and can be assessed in this isolated heart system.

A large body of research, conducted primarily at the Naval Blood Research Laboratory, has demonstrated that red blood cells can be modified to release more oxygen as they pass through tissues by alteration of the red blood cell 2,3-diphosphoglyceric acid content. The ability of such altered red blood cells to deliver increased oxygen has been demonstrated, but the physiologic importance of such increased oxygen delivery on the function of oxygen-dependent organs like the heart has not been definitely established, nor has the importance of such altered oxygen delivery during the shock state been evaluated. The importance of physiologic variables such as blood flow, blood pH, pCO_2, hematocrit, red cell affinity for oxygen, temperature, and composition of the suspending medium (low molecular weight dextran, other osmotically active substances, substrate level and composition) and their effect on optimum myocardial function has been difficult to study because of the large number of variables which can influence cardiac function.

In the Cardiac Muscle Research Laboratory at the Boston University School of Medicine we have devoted a considerable effort in developing an isolated cardiac experimental test system where the independent factors which affect cardiac function can be accurately assessed. We have developed a stable isolated heart preparation where we can control the variables which affect cardiac performance. The many variables which can affect contractility are held constant so that the constituents of the coronary artery perfusate, such as red blood cell oxy-hemoglobin affinity state, are the only experimental variables. This model has been used to study the response of the heart to diffuse ischemia (such as exists in the shock state), hypoxemia (which occurs in the shock state when lung damage prevents adequate oxygenation of the blood), and a variety of metabolic interventions which have the potential to protect the oxygen-deficient myocardium. These studies have shown that the myocardium in the shock state is exquisitely sensitive to small increments in oxygen delivery or alterations in glycolytic metabolism.

This research project brings together the expertise of the B.U. Cardiac Muscle Research Laboratory regarding the use of experimental models which evaluate cardiac function in an experimental shock-state, and the Naval Blood Research Laboratory with its expertise in red blood cell preservation and experimental oxy-hemoglobin modification which improve red blood cell oxygen delivery.

3. Methodology

Isolated Buffer-Perfused Heart Preparation

An isolated isovolumic working rat or rabbit heart preparation has been utilized to perform our research to date. In this preparation, a small cannulated fluid-filled balloon is placed in the left ventricle of the isolated heart and attached to a pressure transducer to monitor intraventricular pressure. Since the balloon is noncompressible, contraction is isovolumic. Because intraventricular volume is held constant, "preload", or diastolic fiber length, does not change; developed pressure and its first derivative (dP/dt) therefore reflect the contractile state of the myocardium.

Surgical and Perfusion Techniques

Albino Sprague-Dawley male rats weighing between 200-300 grams or 2-3 lb. New Zealand albino male rabbits were decapitated and the thorax rapidly opened. The aorta was dissected free, an incision made at the level of the right innominate artery, and a cannula was tied into the root of the aorta. Retrograde coronary perfusion was immediately started via a perfusate reservoir at a level of 75 mm Hg above the heart; thus coronary perfusion was maintained while the heart was being removed from the animal and only a few seconds elapsed between the time of decapitation and the onset of coronary perfusion.

The perfusate consisted of modified Krebs-Henseleit buffer: 118 mM NaCl, 4.7 mM KCl, 2.0 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 0.4 mM Na_2EDTA, 5.5 mM glucose, and 1.0 mM Na lactate. Lactic acid was neutralized with NaOH before being added to the buffer. The lactate was added to the perfusate so that myocardial lactate production or extraction could be measured.

While the coronary bed was being perfused from the fixed-pressure reservoir, the heart was dissected from the thorax. The left ventricle was immediately decompressed by an apical puncture so that a minimum amount of left ventricular contractile work was done during the dissection process. A drain was placed in the apex of the left ventricle so that it remained free of intra-left ventricular fluid from Thebesian drainage. The left atrium was removed and a collapsed latex balloon was inserted into the left ventricular chamber.

The heart was then removed from the thorax and placed in a water-jacketed, constant temperature chamber which was kept at 37°C

with a circulating pump. Coronary perfusion was then switched to a constant flow pump (Harvard Apparatus Model No. 1203 or Technicon Instruments Proportioning Pump Model No. 1). A pacemaker wire was inserted into the right ventricle via a right atrial incision. A polyethylene cannula was inserted into the pulmonary artery to collect the right ventricular ejectate which consisted of coronary sinus drainage, since there was no other flow through the right side of the heart. Some of the efflux drained via the cut vena cavae; this was collected and pooled with the pulmonary artery efflux for metabolic measurements. Only samples drawn from the cannulated pulmonary artery were used for the measurement of the coronary venous pO_2 (Figure 1). The perfusate efflux from the heart was completely collected after one passage through the heart and was not recirculated.

Measurement of Mechanical Function

Ventricular pressures were recorded via a 30 cm length of polyethylene tubing with an internal diameter of 0.045 in. and outside diameter of 0.062 in. (Intramedic Polyethylene Tube PE 160, Clay Adams, Inc., New York, N.Y.).

The frequency response of the pressure recording system was determined by the method of Fry with the balloon attached to the catheter and filled with a volume in the range used during the experiments. The waveform produced by a sudden distortion and release of the balloon resulted in a 16% "overshoot" with after-vibrations of 47 Hz. The damping ratio was 0.54, and calculated natural resonant frequency was 75 Hz. Thus, the system was "critically damped" and the amplitude of the recorded pressure should accurately reflect the amplitude of the true pressure between 0 and the natural resonant frequency or 75 Hz. Rat hearts were paced at 300/min (5 Hz) and it is possible that a minor frequency component of the pressure curve may have been greater than 75 Hz, in which case our recording system would tend to underestimate it. This would be reflected in an underestimated ventricular maximum dP/dt measurement since the most rapid rise of the pressure curve would reflect the highest frequency components. However, even though the absolute maximal ventricular dP/dt may be slightly underestimated in this recording system, since the same technique was used in all hearts, comparisons before and after ischemia in the same heart, and between groups of hearts, should be valid. Furthermore, because the pacing rate was maintained constant at either 3 Hz (rabbit hearts) or 5 Hz (rat hearts) throughout all experiments, the major frequency components of the ventricular pressure trace

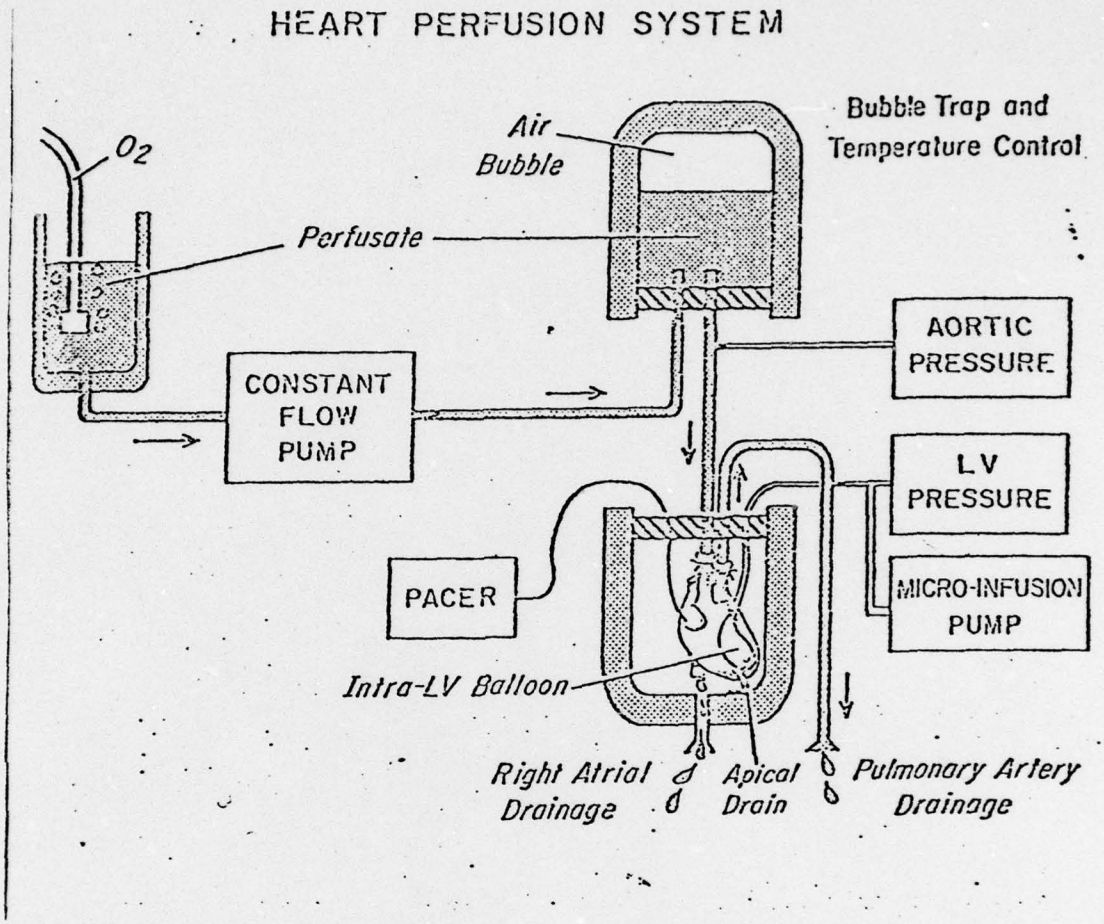


Figure 1. Isovolumic Working Heart Perfusion Apparatus. Perfusate is delivered to the cannulated aortic stump via a constant flow pump which thus controls the coronary flow rate. Since the aortic valve is closed, all perfusate flow is diverted into the coronary arteries. A thin-walled latex balloon fills the left ventricular cavity and is attached to a pressure transducer. Left ventricular volume is determined by the balloon volume which is held constant during the experiment. An apical left ventricular drain allows for the escape of any Thebesian drainage and ensures that the intra-ventricular volume is determined solely by the volume of the balloon. Heart rate is controlled with a right ventricular pacing electrode. Coronary venous efflux is collected via the right atrium and pulmonary artery.

should not have changed relative to the natural resonance of the recording system.

The polyethylene tubing from the ventricular balloon was attached to a Statham P23Db pressure transducer. A photographic recorder with a high frequency response was utilized (Electronics for Medicine Model DR8 or Hewlett Packard Model 4560). Left ventricular dP/dt was obtained via the differentiator output circuit of the Electronics for Medicine SGM Strain Gauge Meter/Amplifier or with an RC differentiator circuit manufactured in our laboratory for use with the Hewlett Packard pressure carrier amplifier Model No. 760-3000.

The collapsed intraventricular balloon was slowly filled with fluid via a precision micro-pump (Harvard Apparatus Model No. 1100) while left ventricular pressures were recorded. The volume of the balloon was adjusted to give a peak left ventricular systolic pressure of 60-80 mm Hg, with a diastolic pressure less than 12 mm Hg. Hearts which could not achieve this level of performance were discarded (approximately 10% of the preparations). Left ventricular pressure and dP/dt were monitored continuously throughout each experiment.

The relationship between balloon size and left ventricular size is critical in this perfusion technique. The balloon must be slightly more capacious than the ventricle, or else, as the balloon is filled, a rise in intra-balloon pressure will be recorded due to increasing balloon wall tension rather than to ventricular wall tension. A series of balloons of slightly different size was manufactured so that in each experiment the ventricular cavity was always slightly less than the balloon capacity. The capacity of each balloon was measured by recording the pressure-volume filling curve of the isolated balloon; the experiments were always performed on the flat portion of the balloon's pressure-volume curve.

The performance of six consecutive rat hearts perfused under well-oxygenated conditions is shown in Figure 2. Hemodynamic performance was stable for the 120 min period. The coronary vascular resistance increased slightly as manifested by a rise in mean aortic pressure at the constant coronary flow rate. This phenomenon has been attributed to coronary arteriolar "autoregulation" in response to a high coronary sinus pO_2 in this preparation. In this series of six hearts the coronary sinus pO_2 was 142 ± 3 mm Hg. Despite the slight rise in coronary resistance over the 120 min period, left ventricular pressure development and maximum left ventricular dP/dt were constant. There was an average 10% extraction of the 1.0 mM arterial lactate, indicating aerobic tissue metabolism.

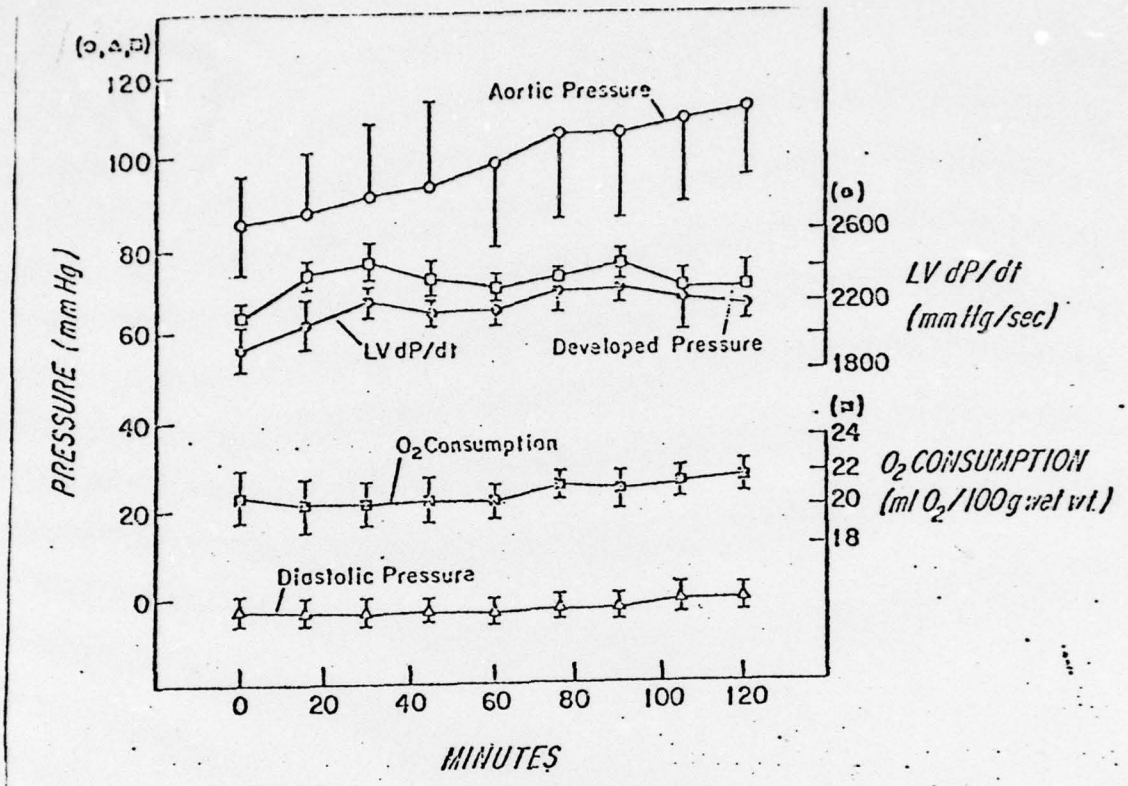


Figure 2. Performance of the Perfused Rat Heart Under Control Conditions. Six consecutive hearts were perfused for 120 min at 37°C with a constant coronary flow of 8 ml/min, perfusate pO_2 = 600 ± 10 mm Hg. Glucose (5.5 mM) and lactate (1.0 mM) were provided as substrate in the Krebs-Henseleit buffer. At the start of the experiment, the left ventricular balloon was filled to produce a left ventricular developed pressure of 60 - 80 mm Hg. The left ventricular balloon volume was not adjusted thereafter. Developed pressure, the maximum rate of rise of left ventricular pressure (left ventricular dP/dt), left ventricular diastolic pressure and myocardial oxygen consumption were stable for the 120 min period. Mean aortic pressure showed a greater variation among the hearts and a slight upward trend, indicating a progressive increase in coronary vascular resistance during the course of the perfusion; this phenomenon has been attributed to "autoregulation" which is secondary to the relatively high coronary venous pO_2 in this system. Similar results have been obtained with the isolated rabbit heart preparation, however, cardiac performance in the buffer-perfused rabbit heart is stable for 270 min. Data all plotted as ± SEM.

Experimental Shock (Ischemia) State

A number of mechanical, electrophysiologic, and biochemical parameters need be measured, both during a period of ischemia and after reflow and return to control coronary flow rates. It is important to assess function both during the ischemic and reflow periods because a number of interventions have been shown to be ineffective in improving myocardial function during the period of ischemia or hypoxia when function is severely depressed; however, during a reflow or reoxygenation period, the beneficial or deleterious effects of these interventions become apparent (See Apstein et al, J. Molec. and Cell. Cardiol., 8: 627-640, 1976, and Apstein et al, Circulation 55: 864-872, 1977). Furthermore, in many clinical situations, one of the goals of therapy is to preserve myocardial function while awaiting improved coronary perfusion or oxygenation which may be brought about acutely by correction of systemic arterial hypotension or arterial hypoxemia. Hence, in addition to any immediate change in ischemic cardiac performance, preservation of recoverability is also a parameter which an experimental system should test. The experimental model should be designed so that the coronary perfusate can be readily switched from one substrate to another to aid in the study of metabolic, pharmacologic, or hematologic factors.

In order to assess the effects of various interventions on ischemic performance or recovery from the shock state, it is important to accurately measure contractile performance. Ideally, the "preload" and systolic "after-load" should remain constant during an experiment, so that they do not influence left ventricular pressure development.

Furthermore, it is necessary to control the amount of myocardial oxygen demand in the control state so that a given level of ischemia causes a constant reduction in oxygen supply relative to a constant oxygen demand; ischemic injury might be more severe if a given reduction of oxygen delivery occurred in circumstances where oxygen demand was greater. The degree of ischemia should be held constant during an experiment or performance will vary on this basis alone.

For the reasons cited above and because of the importance of maintaining coronary flow constant at a precise ischemic level, we have adopted a methodology which utilizes a constant flow pump to provide coronary perfusion, in the constant coronary flow, isovolumic, isolated working rat or rabbit heart preparation as originally described by Fallen et al (J. Appl. Physiol. 22: 836-839, 1967) and adapted in our laboratory, as described

above, and in two recent publications (Apstein et al, Graded Global Ischemia and Reperfusion: Cardiac Function and Lactate Metabolism. Circulation 55: 864-872, 1977; Apstein et al, Ventricular Contracture and Compliance Changes with Global Ischemia and Reperfusion, and Their Effect on Coronary Resistance in the Rat. Circulation Research, 41: 206-217, 1977). Basically, we have adapted the preparation of Fallen et al (J. Appl. Physiol. 22: 836, 1967) and adjusted the experimental ischemic coronary flow rates to values on a ml/min/gm basis to produce models of experimental shock.

This preparation provides an experimental model which has stable mechanical and metabolic performance for 120 min in isolated rat hearts; in isolated rabbit hearts performance is stable for 4½ hours. We plan to utilize the isolated rabbit heart in the buffer-perfused isolated heart preparation in all future work because of the longer period of stability of the preparation. We will employ a protocol of 1½ to 3 hours of ischemia (shock state) with one hour of reperfusion.

This is an important advantage since some of the processes which cause ischemic tissue damage are probably time-dependent and may not be observed in a relatively short ischemic protocol. Furthermore, we have demonstrated that the response of the ischemic heart to glucose and insulin is markedly time-dependent (See Results, below); in short protocols (<30 min of ischemia), the beneficial effects of glucose and insulin on the ischemic myocardium were not apparent (probably because the heart has adequate glycogen stores), however, with longer ischemic protocols, achievable only in the isolated rabbit heart preparation, the advantages of hyperglycemia and insulin as substrate became apparent.

The isolated rabbit heart is probably more closely related to the human heart than the rat heart in other ways as well. The isolated rabbit heart beats at a slower rate (in our preparation we pace the rabbit hearts at 180/min and exceed all endogenous pacemakers, while in the rat heart a pacing rate of 300/min is required). The rat heart has a much higher rate of myocardial oxygen consumption, not only because of the higher rate of beating, but also because of the endogenous rate of oxygen consumption. This is probably related to the rat's known high rate of velocity of contraction (Henderson et al, Am. J. Physiol. 217: 1273-1279, 1969) and high rate intrinsic ATPase activity level. (Kleid, J.J. et al, J. Mole. and Cell. Cardiol. 4: 625-632, 1972). In our preparation, the rat heart utilized 24±2 ml O_2/100 gm/min compared to the rabbit heart's oxygen consumption, 8±1 ml/100/gm/min. This lower rate of oxygen consumption by the rabbit heart may account for its longer in vitro stability, and also makes the rabbit myocardium closer to the human myocardium in terms of this parameter.

Furthermore, the rabbit heart is technically easier surgically and in terms of instrumentation because of its larger size (approximately 2.5 gm of left ventricle for the rabbit vs. 0.5 gm of LV for the rat heart). Thus surgery is easier and it is even possible to suture an epicardial length gauge on the rabbit LV.

In addition, we are performing ultrastructural analysis and tissue biochemical assays of cytoplasmic enzymes and high energy phosphate levels (see below). The rabbit heart has a distinct advantage over the rat heart; the greater mass of myocardial tissue allows multiple assays to be performed from a single heart. Thus, for future studies, we plan on utilizing the isolated rabbit heart exclusively in the isolated buffer-perfused heart preparation.

Assessment of Cardiac Function and Cellular Integrity. The isolated isovolumic perfused heart preparation which will be utilized in this project allows for precise measurement of myocardial performance. Cardiac function will be assessed by measuring a number of parameters:

1. Peak left ventricular systolic pressure (since contraction is isovolumic and "preload" is held constant in this preparation, peak LV pressure reflects the inherent contractile state of the muscle).

2. Left ventricular positive dP/dt.

3. Isovolumic diastolic ("contracture") pressure and diastolic compliance.

4. Rate of ventricular relaxation (negative dP/dt).

5. Coronary vascular resistance.

6. Arrhythmias as monitored from epicardial electrodes.

7. Pacing threshold.

Measurements 1-6 will be made continuously during the course of an experiment. The pacing threshold will be determined every few minutes.

In this preparation there is no ventricular filling since the non-compressible, isovolumic, fluid-filled balloon occupies the left ventricle. Therefore, diastolic ventricular pressures reflect one point on the diastolic compliance curve. We have recently demonstrated that a shift in this point, i.e. development of "contracture," accurately reflects the change in the passive ventricular compliance curve over the physiologic range of filling pressure (0-30 mm Hg). (See Apstein et al, Ventricular Contracture and

Compliance Changes with Global Ischemia and Reperfusion, and Their Effect on Coronary Resistance in the Rat, Circ. Research, 41: 206-217, 1977.) Thus, our continuous monitoring of LV diastolic pressure in this preparation provides us with a continuous assessment of diastolic ventricular compliance.

Coronary vascular resistance is measured by monitoring the aortic cannula perfusion pressure at a constant or known coronary flow rate (see Figure 1 and Circ. Res. 41: 206-217, 1977).

Biochemical and Metabolic Assessment. The metabolism of the working heart is evaluated by sampling the arterial perfusate and venous efflux during the experiment. Determinations of oxygen and substrate uptake (or metabolite production) will be done by measuring the arteriovenous concentration differences. Since the coronary flow rate is accurately known and maintained constant during an experiment, metabolic consumption or production rate can be readily calculated.

Metabolic monitoring during an experiment consists of determination of oxygen consumption, and lactate extraction or production. At selected times the heart is rapidly frozen with a clamp cooled to the temperature of liquid nitrogen (Wollenberger clamp) and measurements of tissue levels of glycogen, ATP and creatine phosphate are performed as described by Weissler et al (J. Clin. Invest. 47: 403, 1968). Lactate is measured by the automated enzymatic method developed by the project director (Apstein et al, Anal. Biochem. 38: 20-34, 1970). Tissue calcium is measured by atomic absorption using the method of Willis, J.B. Determination of calcium and magnesium in urine by atomic absorption spectoscopy. Anal. Chem. 33: 556-559, 1961. Other metabolites may be measured in specific protocols. Tissue edema is measured by measuring the wet:dry weight ratio and also by measuring the tissue insulin space. Tissue metabolite will be expressed per gm dry weight.

We will also monitor epicardial fluorescence at appropriate wavelengths to measure the redox state (NAD/NADH ratio). Measurement of epicardial fluorescence in buffer-perfused hearts eliminates interference with red cell NADH content and has been done in several labs. (Lai and Scheuer, J. Molec. Cell. Cardiol., 7: 289, 1975; Chance, Williamson et al, Biochem. Zeit., 341: 357, 1965; Chance et al, Am. J. Physiol., 223: 207, 1972). Epicardial fluorescence can also be measured in blood-perfused hearts, but is more difficult. (Barlow et al, "Evaluation of ischemic areas in cardiac tissue by fluorescence spectroscopy." Presented at American Section of International Society of Heart Research

Meeting, Pasadena, California, May 1977). The measurement of epicardial fluorescence gives a direct measure of the redox state; knowledge of this parameter, and changes which occur with any intervention, is critical to understanding ischemic dysfunction and the role of increasing oxygen delivery.

A tissue aliquot is also assayed for tissue creatine phosphokinase (CPK) activity. Washout of CPK will be used as a biochemical index of tissue damage. The extent of myocardial CPK depletion has been shown to linearly correlate with the morphologically assessed size of experimental myocardial infarction (Kjeksus, J.K. and Sobel, B.E., Circ. Research 27: 403-413, 1970). In the anoxemic perfused rat heart significant CPK leakage occurs after 20 minutes of oxygen deprivation; the extent of CPK release correlates with depression of high energy phosphate stores and loss of recoverability of mechanical function (Hearse, D.J. and Chain, E.D., Recent Adv. in Studies in Cardiac Structure and Metabolism, Vol. 3: Myocardial Metabolism. Ed. by Dhalla, N.S., University Park Press, 1973). Therefore, the extent of CPK washout appears to be a valuable biochemical marker for assessing ischemic damage in our proposed methodology. CPK will be measured by the method of Rosalki (J. Lab. Clin. Med. 69: 696, 1967) and expressed as units per mg of myocardial protein.

Current operational biochemical methods in the Principal Investigator's lab include the tissue ATP, CP, lactate, and CPK methods noted above; a pO_2 electrode is currently routinely used to determine oxygen consumption. An epicardial fluorometer is currently under construction. The tissue calcium method is currently operational in a collaborating laboratory.

Ultrastructural Assessment. Because electron microscopy examines a very small tissue sample, the possibility of sampling error is large. In order to minimize this problem, we have adopted the following protocol. Only hearts which are representative of an experimental group are selected for electron microscopy. The mechanical performance of an experimental group will be defined by perfusing 16 hearts (8 without reperfusion, 8 with reperfusion). Hearts will then be perfused for fixation for electron microscopy, but they will only be accepted for electron microscopic evaluation if their mechanical performance is within one standard deviation of the mean of the entire group. In other words, hearts which perform at the best or worst end of the range of performance for an experimental group will not be used for electron microscopic evaluation. This will ensure that ultrastructural assessment will only be done in hearts representative of each group. Each heart will have four tissue blocks sampled and ultimately sectioned, one from each of the four walls of the ventricle. The tissue blocks will be taken from the mid-myocardial level to avoid endocardial

damage resulting from the intra-ventricular balloon or epicardial damage resulting from direct trauma during the experimental manipulation. Ten to twelve representative micrographs at a constant level of magnification will be taken from each tissue block. The electron microscopic technician who performs this work will receive coded hearts and not know the experimental protocol for each heart. Thus, each heart will generate approximately 40-50 micrographs and a series of 3 hearts approximately 150 micrographs. These will be coded and numerically graded by several investigators (including the project director) according to pre-established criteria. The ultrastructure interpreters will not know to which protocol the hearts were subjected, and they will grade the micrographs independently of each other.

We have developed a set of "reference micrographs" which each grader will use to quantify the degree of ischemic ultrastructural damage. Different degrees and duration of ischemia were inflicted in a series of hearts in order to create a range of ischemic damage. Study of these micrographs led to the development of three separate sets of criteria of morphologic damage by independently evaluating the mitochondria, sarcomere structure, and intracellular edema or T-tubule system swelling. A set of reference photographs was generated and these reference photographs will be used by all ultrastructural graders in determining tissue damage in the coded micrographs in which emanate from the experimental protocols. The reference micrographs for damage are attached as Figures 3-6 of this application.

This approach should yield an objective quantification of ultrastructural damage with the selected interventions and also permit us to determine whether a given intervention has a specific effect on the mitochrondrial, or sarcomere damage, or on intracellular fluid accumulation.

Each electron micrographic grader will give each micrograph a "point score" for each set of damage criteria. The scores will be averaged for each heart by averaging the scores of the four walls and different groups of hearts will be compared utilizing the unpaired Student t-test.

Tissue Fixation and Preparation for Electron Microscopy

Fixation and Dissection. At the termination of the experiment the heart is perfused with a mixture of 1% glutaraldehyde and 4% formaldehyde in phosphate buffer pH 7.4, 200 mOsm for 30 min. The specimen is then preserved in the same solution at 4°C. until dissection. Specimens are taken from four areas of the left

ventricle at a mid-level between base and apex: lateral, anterior, posterior wall and septum. The specimens consist of 2 x 1 x 1 mm samples of longitudinally oriented myocardial fibres out of the midzone between endocardium and epicardium.

Dehydration and Embedding. The specimens are postfixed in OsO_4 1% hydrous solution for 90 min., stained en bloc with 0.1% uranyl acetate, and dehydrated through 75, 95, 100% ethanol, followed by 100% propylene oxide. The infiltration with 1:1 mixture of propylene oxide and epon lasts at least 90 min, with continuous movement. The specimens are then placed in epon (epon 812 48 ml, NMA 28 ml, DDSA 24 ml, DMP-30 2 ml), and cured in flat embedding silicone molds for 24 hours at 60°C.

Sectioning and Microscopy. One-micron sections are cut with glass knives and stained with Azure II-methylene blue. On the basis of these light microscopic sections, areas with longitudinal orientation of myocardial cells are selected and the blocks are trimmed for electron microscopy accordingly. These sections are cut with a diamond knife on an LKB II ultramicrotome and placed on a 200 mesh copper grid covered with a parlodion-carbon film. These grids are then stained with uranyl acetate and lead citrate. Multiple electron micrographs are taken with a Phillips EM 300 at 80 KV at standard magnifications of 3200 and 8100. Contact prints are used to evaluate 10-12 electron micrographs from each area (40-50 per heart). In addition, the area covered by the EM section is compared with the original light microscopic preparation. The extent of the inter-cellular edema is estimated, and the distribution ofcellular changes, as seen in the EM, is assessed on the larger area, as far as visible with the resolution of the light microscope.

The electron microscopic evaluation is done in the laboratory of Dr. Christian Haudenschild.

Correlations of Separate Components of the Ischemic Injury Process

A flow sheet summarizing the general protocol to be followed in the globally ischemic, perfused heart is shown in Table 1. The ischemic coronary flow rates and length of ischemic periods are designed to induce shock damage which depresses recovery by 50%; thus, there is room for improvement, but the tissue is not so severely damaged as to preclude any improvement of recovery.

This is an important experimental point; if the shock damage is not severe enough, then recovery may be virtually complete without any further intervention or treatment. Contrariwise, if the induced damage is too severe then recovery or improvement may be impossible.

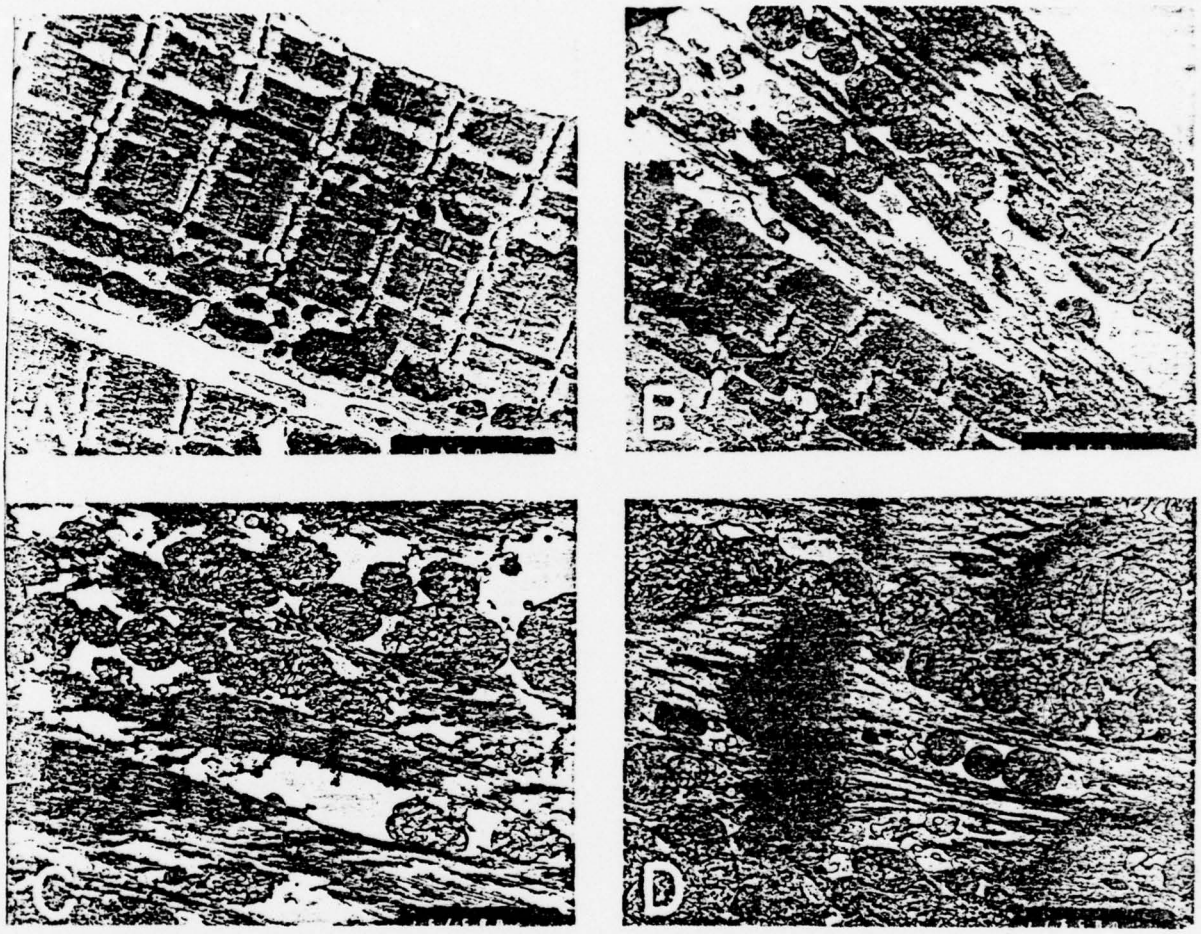


Figure 3. Reference Micrographs for Sarcomere Damage.

A. Grade 0 sarcomere damage or normal sarcomere structure. Note that the Z lines are in register and the the I bands, A bands, and the H zone with M and L bands are clearly visible.

B. Grade 1 sarcomere damage is shown in the sarcomeres of the lower left quadrant of the micrograph. The Z lines are out of register between sarcomeres, and within a given sarcomere there is distortion of the Z line. There is a slight loss of intra-sarcomere structure with obliteration of the H zone and loss of definition of the junction between the I band and A band.

C. Grade 2 sarcomere damage. Although a sarcomere unit can be recognized, there is obliteration of the normal A, I and Z band structures. The Z band appears to be replaced by a thick band of material which may represent "contracture bands" and contractile proteins which have pulled free of their normal intra-sarcomere attachments.

D. Grade 3 sarcomere damage. There is complete obliteration of sarcomere structure with large lumps of densely staining material which appear to be contractile protein which has pulled completely free from its normal linear relationship. Thin filamentous strands of pre-existing sarcomere structure can be seen, but no sarcomere units can be distinguished.

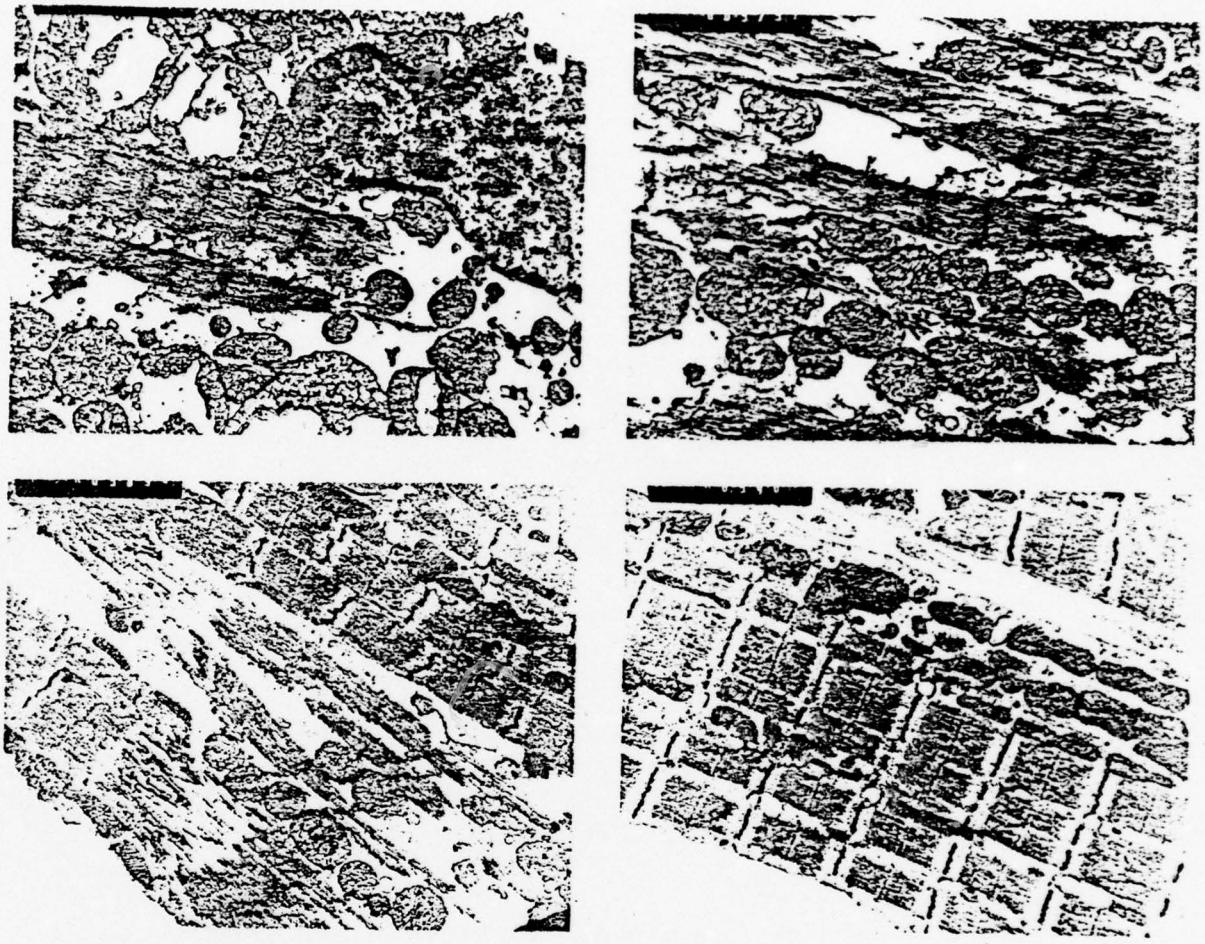


Figure 4 . Reference Micrographs for Mitochondrial Damage.

A. Grade 0 mitochondrial damage (normal). The mitochondria are cigar-shaped, densely staining, and have clearly visible parallel internal membranes (cristae).

B. Grade 1 mitochondrial damage. The mitochondria are spherical and slightly swollen. Intra-mitochondrial fluid increase is visible with small vacuole formation and slight disorganization of the internal membranes.

C. Grade 2 mitochondrial damage. There is a moderate swelling of the mitochondria with further disruption of internal membranes and accumulation of intra-mitochondrial fluid.

D. Grade 3 mitochondrial damage. There is swelling of mitochondria with lakes of intra-mitochondrial fluid, and fragmentation of internal and external mitochondrial membranes. This micrograph also demonstrates the variable nature of mitochondrial damage on a single section, as some mitochondria are clearly more damaged than other.

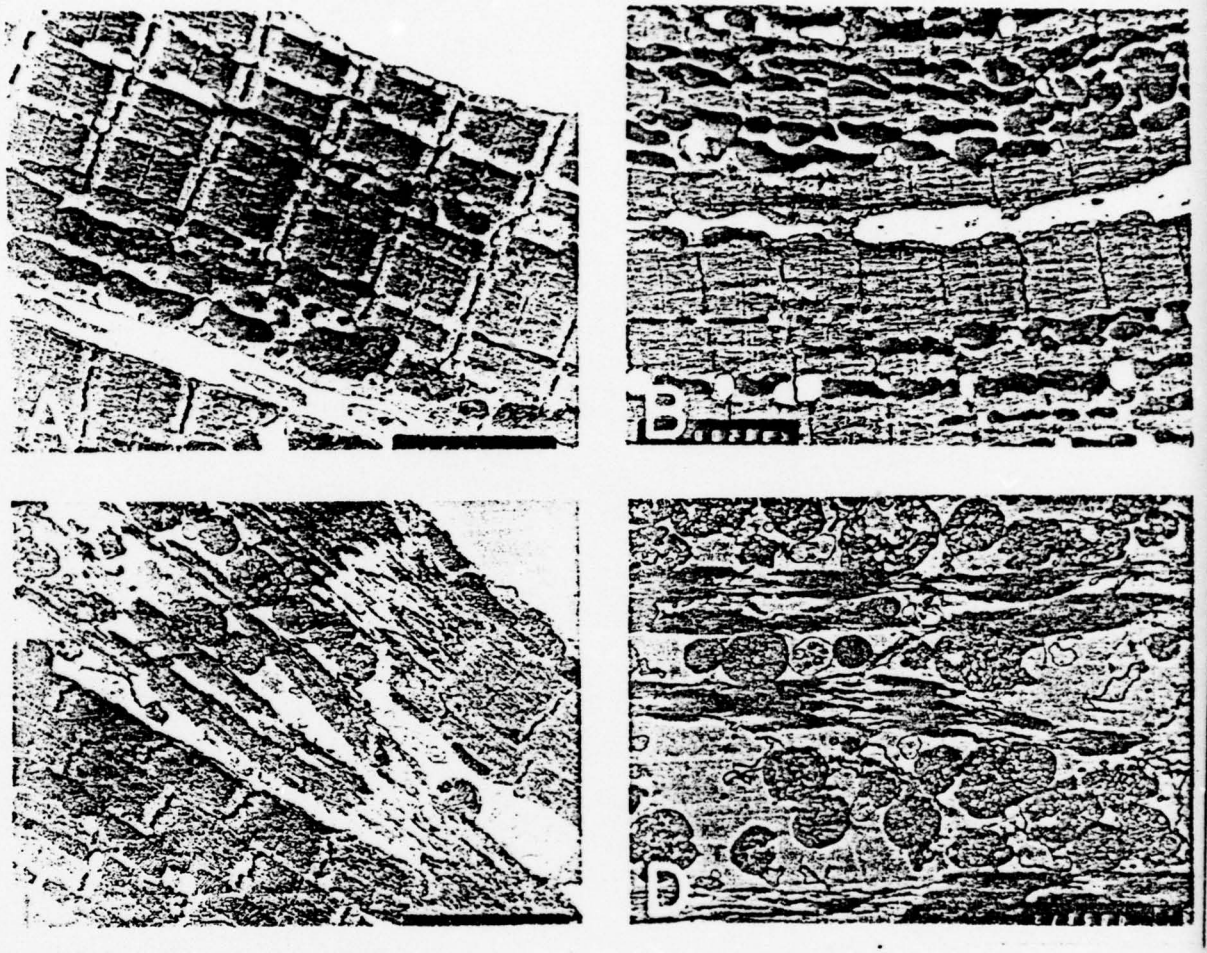


Figure 5. Reference Micrographs for T-tubule Swelling and Intracellular Edema.

A. Grade 0 damage (normal). There is no intracellular edema; normal T-tubules are seen in cross-section.

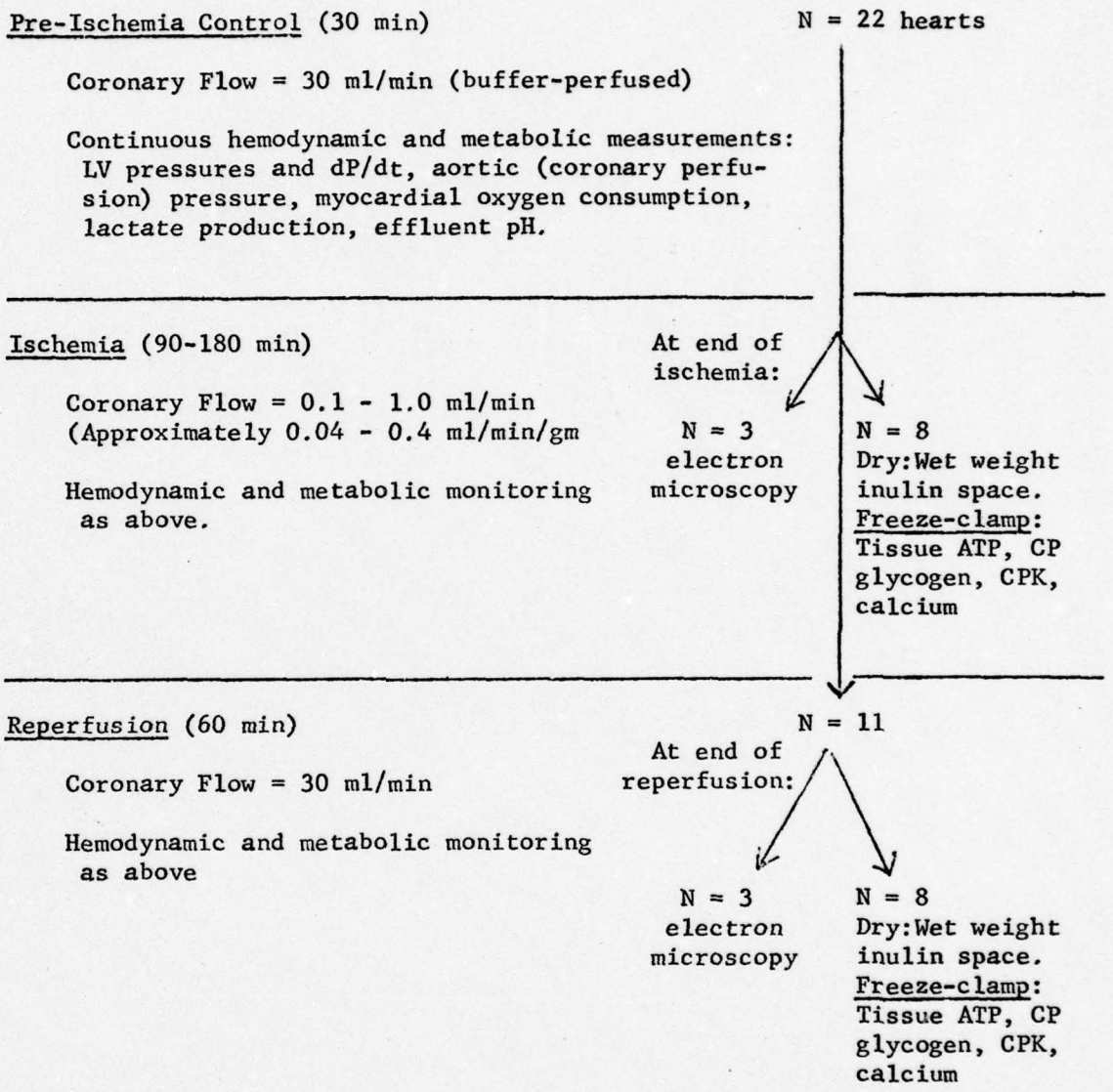
B. Grade 1 damage is shown in the lower half of the micrograph. There is swelling of the T-tubules in cross-section. However, no intracellular edema is apparent.
Grade 1.5 damage is shown in the upper half of micrograph B; peri-mitochondrial edema fluid has become evident.

C. Grade 2 damage. There is T-tubule swelling and, in addition, focal areas of intracellular edema.

D. Grade 3 damage. Large areas of intracellular edema are seen.

TABLE 1

General Experimental Protocol for Shock State (Ischemia)



Alteration of RBC Suspending Medium: Effect of Recycling, Colloid Content of Perfusate, Hyperglycemia, Insulin, Mannitol

Recirculation of the Perfusate. In our original buffer-perfused system large volumes of Krebs-Henseleit buffer perfusate were used and discarded after one passage through the heart. In a five hour experiment, approximately 10 liters of perfusate are required for the isolated rabbit heart. Clearly, this is an impractical and impossible perfusion system for a blood-perfused heart and we have modified our system to trap the perfusate after it passes through the heart and recirculate it. With our current experimental apparatus the minimum volume required is approximately 500 ml of perfusate or approximately 1 unit of whole blood.

A large part of the required blood volume (200-300 ml) was necessary to prime the membrane oxygenator which was initially used in the system. Subsequently we have designed our own oxygenator. The "oxygenator" consists of 10 ft. of thin-walled silastic tubing which is placed in a beaker and gassed with an appropriate O_2 level, depending on the desired O_2 loading condition. The dead space of this "lung" is only 5 ml, and it costs approximately $2 vs. $80 for the membrane oxygenator. Oxygenation to a pO_2 of 600 can be achieved with the flow rates required for our protocol.

We have performed a series of pilot experiments where our standard Krebs-Henseleit buffer perfusate was recirculated through the heart for a period of five hours. In contrast to our previous non-circulating experiments, the cardiac performance in the recirculating system was not stable over the five hour experimental protocol but declined after 1-2 hours. Subsequent metabolic analysis ot the perfusate revealed that the perfusate glucose and lactate levels progressively decreased as they continuously recirculated through the heart. Consequently, at the end of the five hour period, the substrates decreased to about 60% of control for glucose and less than 25% of control for lactate.

Accordingly, we developed an "enriched" Krebs perfusate consisting of 200 mg % glucose and 5 mM lactate. These substrate levels appear to be adequate (see Results).

Addition of Albumin to the Perfusate. We have previously reported that the isolated heart, perfused with Krebs-Henseleit buffer becomes progressively edematous over the five hour experimental period. The edema is intercellular (as opposed to intracellular) and does not impair contractile function. However, it is likely that with a blood perfused system this intercellular edema would compromise the capillary lumen space and increase resistance to blood flow. Therefore, it is important to reduce the tissue edema which occurs in this preparation if the blood

perfused heart is to be successful. Accordingly, we have performed a pilot series of experiments adding 6% albumin to the perfusate to increase the colloid osmotic pressure of the perfusate to the normal range for the rabbit. The addition of this protein to the perfusate required that we change our oxygenator to a membrane oxygenator. This was successfully done and we can now achieve an arterial pO_2 greater than 600 mm Hg at our required flow rate of 30 ml/min. However, initially, despite this adequate pO_2 we have not been able to get as good contractile function with the albumin perfusate as we have previously obtained for our albumin-free perfusate.

There were two likely causes for the depressed performance with the albumin perfusate. First of all, albumin binds calcium. Since free or ionized calcium directly affects contractility it is likely that the depressed level of contractility is due to a low ionized calcium concentration of the albumin perfusate.

Secondly, it is possible that there are contaminants in our commercially prepared albumin which may depress cardiac function. Accordingly, the albumin perfusate preparation was first dialyzed for 24 to 48 hours in the cold against a large volume of buffered albumin-free perfusate.

Adjustment of the ionized calcium level and dialysis of the albumin has resulted in an isolated heart preparation which achieves normal function and is stable for several hours (see Results).

Effect of Glucose Level, Insulin, Mannitol

The red blood cell suspending medium composition was assessed for a possible independent effect on normal and ischemic cardiac function, and also to determine whether our methodology was sensitive enough to detect an improvement in myocardial preservation during the shock state.

Different groups of rabbit hearts were subjected to two degrees of ischemia and perfused with Krebs-Henseleit buffer solutions which contained several different substrates. The length and degree of ischemia was designed to induce approximately a "50% lethal injury," i.e. reduce recovery by 50%, so that improvement or worsening was possible. Accordingly, the control coronary flow rate of 30 ml/min was reduced to 1 ml/min for 180 min, to induce "moderate" ischemia, or 0.1 ml/min, to induce "severe" ischemia. The perfusate substrates were either (a) normal glucose (100 mg % or 5.5 mM), (b) 5x normal glucose (500 mg % or 27.5 mM) + 100 mU/ml insulin, or (c) normal glucose + 400 mg % mannitol. The mannitol served as an "osmotic control" since it duplicated the increased osmotic pressure of the high glucose

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group, but since mannitol is not metabolized by the heart, it had no metabolic effect.

The results of this series of experiments (see below) clearly demonstrates the importance of the suspending medium as an independent determinant in protecting myocardial tissue during the shock state.

Blood-Perfused Isolated Heart

After acquiring the experience and background with the buffer-perfused heart, as outlined above, we attempted to perfuse the isolated rabbit heart with human red cells.

Washed outdated human RBC which have been preserved to give approximately 80% of the normal 2,3 DPG content are utilized. The packed cells are re-suspended in "enriched" Krebs-Henseleit buffer containing 11 mM glucose, 5mM lactate, 2.5 gm % dialyzed albumin. The ionized calcium content is adjusted to the normal range (approximately 2.5 mEq/L) and monitored throughout the experiment. The hematocrit is adjusted to 30%. The pH is adjusted to approximately 7.75 with 0.1 N NaOH; this alkaline value was empirically utilized since the pH is reduced to approximately 7.40 after passage through the silastic tubing "lung" and equilibration with the 95% O_2-5% CO_2 gas mixture.

Coronary perfusion pressure was maintained at 100 mmHg during the experiment, and coronary arterial flow was allowed to vary and follow the coronary vascular resistance. In general the coronary resistance began to increase after approximately 1½ hours of perfusion as can be seen from the progressive decrease in coronary flow at constant coronary perfusion pressure. The hearts were allowed to beat at their own intrinsic rate so that heart rate could be followed as an index of cardiac performance. In order to compare ventricular pressure development at a common heart rate, a short strip was recorded every 15 min with the hearts paced at 180/min.

Oxygenation was accomplished with the silastic tubing "lung" described above. The blood suspension was passed through a 20 micron filter prior to use and a second 20 micron filter was placed "in-line" in the perfusion apparatus.

We utilized the blood pump designed by Dr. M.A. Castany for perfusion of isolated retinal preparations. Basically the blood bag is compressed in a fluid-filled tank at a constant rate so that blood is forced out without trauma to the red cells by a roller pump.

Using these methods we have been able to achieve stable

performance in the physiologic range for approximately 2-2½ hours (see Results).

Methodologic Problems to be Solved

The stability of the preparation is probably adequate for performing experiments of less than 120 min duration; however, we will attempt to develop a model which can maintain stable function for a longer period of time. We should be able to equal the endurance of the buffer-perfused heart which performs at a stable level for 4½ hours.

It should be noted that the blood suspension used to date contains no platelets, only 50% of the normal albumin level, and outdated washed RBCs with a moderate decrease in the 2,3 DPG level. Accordingly, by modifying one or all of these factors we hope to be able to improve the duration of stable performance of the isolated heart.

A major problem which plagues the blood-perfused experiments is the need for rapid and repeated assessment of the blood electrolyte and ionized calcium levels throughout the day. Currently, we measure the ionized calcium utilizing the Orion calcium electrode; however, this instrument is unreliable and costly to operate in terms of reagents and manpower. We currently do not monitor other blood electrolyes. The calcium-electrolyte monitoring problem could be solved by a new instrument, the Clin-Ion meter which can rapidly measure ionized calcium and electrolytes on repeated samples, however, the instrument costs approximately $10,000.

4. RESULTS

Modification of RBC Suspending Medium: Effect of Recycling With "Enriched" Buffer Perfusate

Increasing the perfusate glucose level to 11 mM and lactate level to 5 mM resulted in stable contractile function for 3-4 hours despite recycling of the perfusate. At the end of the experimental run the recycled perfusate had a residual glucose level of >100 mg % and lactate level > 1 mM indicating that substrate depletion did not contribute to cardiac failure.

Protection of Ischemic Myocardium With Hyperglycemia and Insulin

Our results have demonstrated a definite protective effect of hyperglycemia and insulin on ischemic myocardial tissue. The protective effect was dependent upon the severity of the degree of imposed ischemia and the duration of the ischemic period.

Isolated rabbit hearts were subjected to a 180 min period of moderate ischemia followed by reperfusion, or a 90 min period of severe ischemia followed by reperfusion. Substrate consisted of 100 mg % glucose - no insulin (control) or 500 mg % glucose + insulin, 100 mU/ml. The duration of the ischemic period was chosen so that the control group, with 100 mg % glucose, recovered to approximately 50% of the preischemic developed pressure level. Thus either a beneficial or deleterious effect of a hyperglycemia and insulin substrate could be observed. The moderate ischemia group was perfused at an ischemic coronary flow rate of 1 ml/min or 0.4 ml/min/gm; this represents approximately 16% of the normal myocardial perfusion rate for the rabbit. The severe ischemia group had an ischemic coronary flow rate of 0.1 ml/min or 0.04 ml/min/gm. In the moderate ischemia group the high glucose and insulin substrate was begun after 5 min of ischemia had elapsed; reflow occurred in the presence of the control glucose - no insulin substrate. In the severe ischemia group, the high glucose and insulin substrate was begun 15 min prior to the onset of ischemia; reflow occurred with the control glucose - no insulin substrate.

Developed left ventricular pressure (peak systolic pressure minus diastolic pressure) was unaffected during ischemia by the high glucose and insulin substrate at both degrees of ischemia. However, in the reflow period, the moderate ischemia group which had received the high glucose and insulin substrate during the period of ischemia had a significantly better recovery than the control glucose group. Under conditions of severe ischemia, however,

the high glucose - insulin substrate did not enhance recovery in the postischemic period (Figures 6 and 7).

The presence of hyperglycemia and insulin prevented ischemic contracture during both moderate and severe ischemia Figures 8 and 9). With reperfusion after severe ischemia, intense contracture occurred in the high glucose and insulin group. However, reperfusion after moderate ischemia resulted in only transient contracture in the high glucose and insulin group and return of diastolic pressure to the control level with continued reperfusion. Thus, with moderate ischemia the hyperglycemic - insulin substrate protected the diastolic properties of the ventricle from contracture during ischemia and reperfusion; with severe ischemia diastolic protection was afforded only during the period of ischemia, and with reperfusion severe contracture developed despite the absence of contracture during the ischemic period.

The metabolic consequences of the hyperglycemia and insulin substrate are shown in Figures 8 through 10 and Table 2. With severe ischemia there was a marked increase in the coronary venous effluent lactate concentration in both the control and the hyperglycemia - insulin group. However, after approximately 50 min of severe ischemia the effluent lactate concentration level in the control group began to decrease while the effluent lactate concentration in the hyperglycemia and insulin group progressively increased. Thus, during the last 30 min of the severe ischemia protocol the high glucose and insulin group produced more lactate; during the first few minutes of reperfusion there was more lactate washout from the high glucose and insulin group. A similar pattern was observed with moderate ischemia. (Figure 9). During the initial 30 min of ischemia there was no significant difference between the control and high glucose - insulin groups with respect to the venous effluent lactate concentration. However, after 45 min of ischemic perfusion, the control glucose group demonstrated a progressive decline in effluent lactate concentration while the hyperglycemia - insulin group showed a progressive increase in the venous effluent concentration; during the early reperfusion period more lactate was washed out of the hyperglycemia - insulin group.

In a subgroup of experiments, the hearts were frozen at the end of the ischemic period, prior to reperfusion, and the tissue lactate and ATP levels assayed. The results of the complete anaylysis of lactate metabolism is shown in Figure 10. Under conditions of severe ischemia, the presence of high glucose and

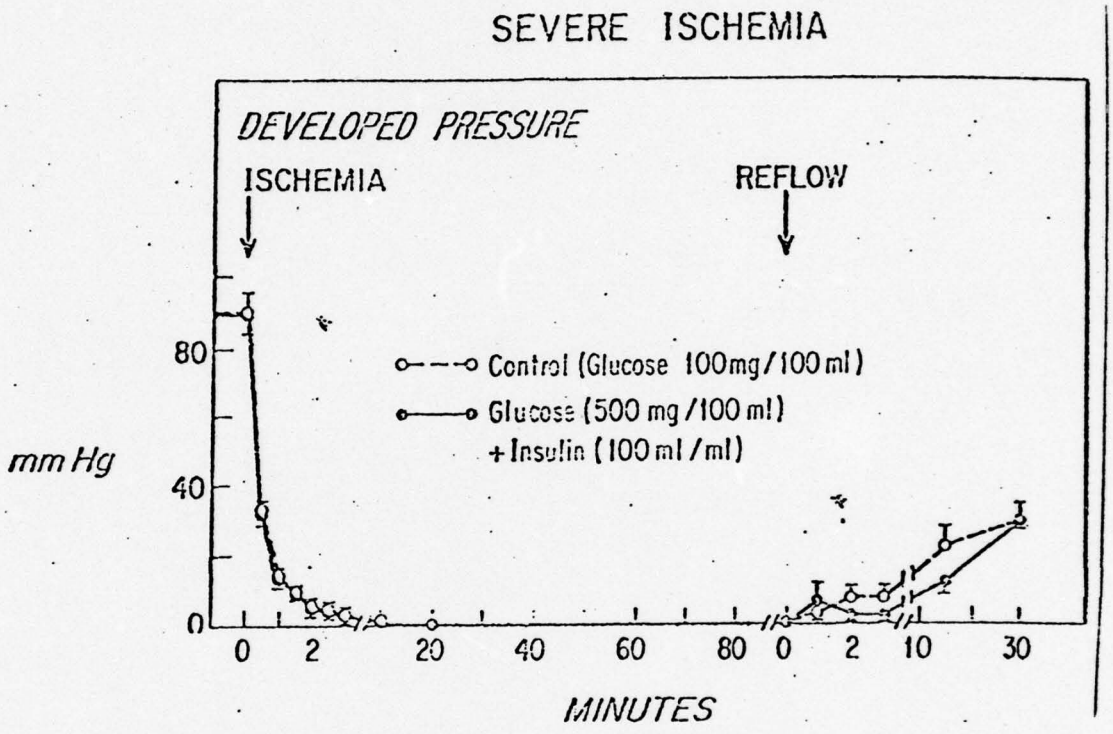


Figure 6. Effect of hyperglycemia and insulin on developed pressure during and after severe ischemia. Ten hearts in each group were perfused at an ischemic flow rate of 0.04 ml/min/gm. There was no significant difference in developed pressure during ischemia or reperfusion.

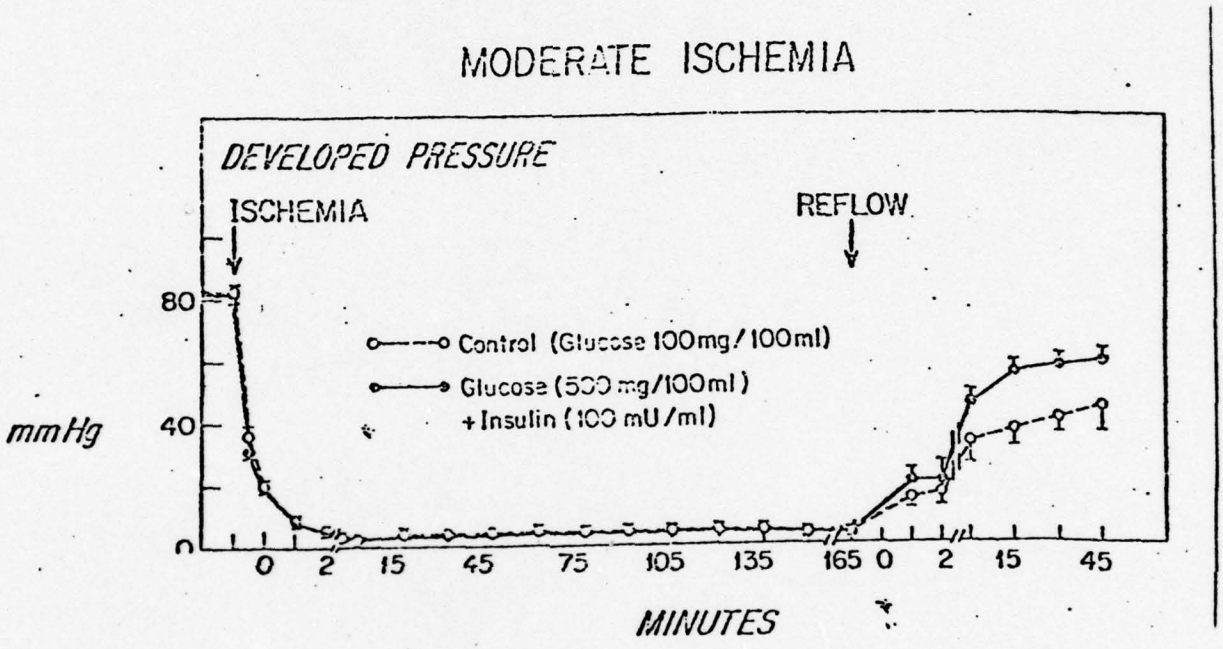


Figure 7. Effect of hyperglycemia and insulin on developed pressure during and after moderate ischemia. Twenty-eight hearts in each group were perfused at an ischemic flow rate of 0.4 ml/min/gm. There was no effect on developed pressure during the period of ischemia; however, during reperfusion, the hyperglycemia and insulin group had a significant better recovery ($p<0.05$ for 15 min to 60 min; 60 min point not shown).

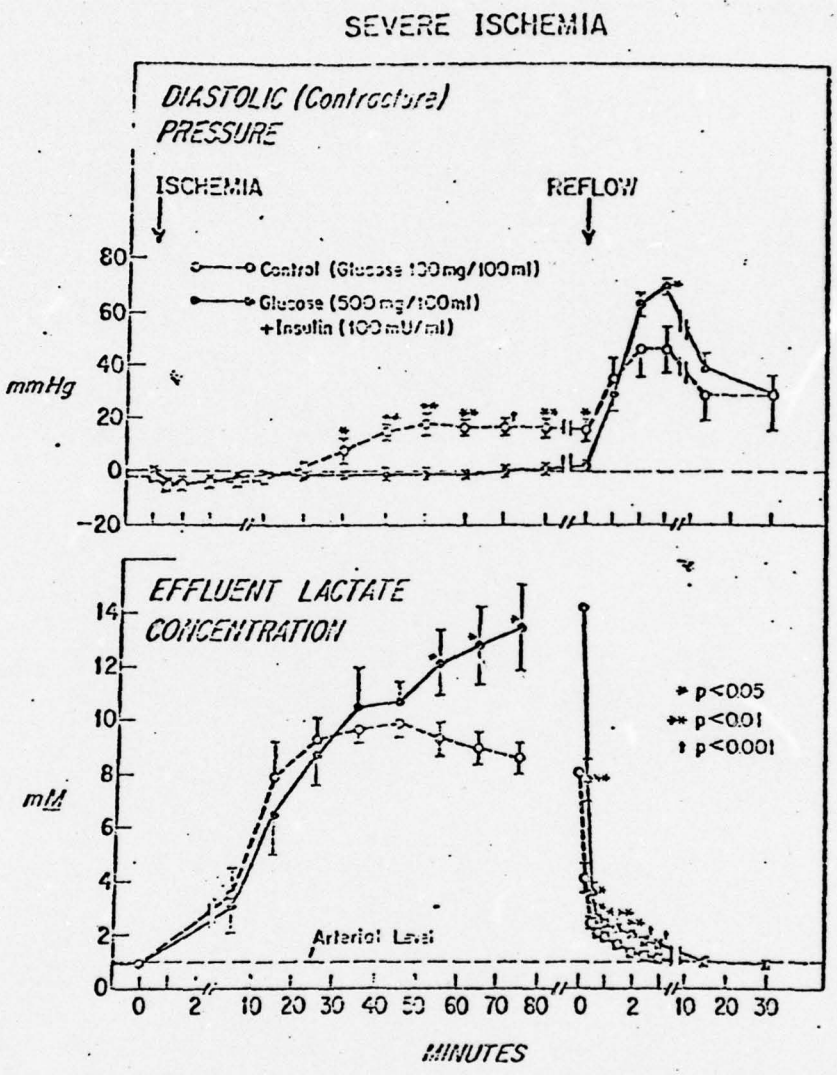


Figure 8. Effect of hyperglycemia and insulin on contracture and lactate production during severe ischemia. (See text for discussion.) (n=10 in each group)

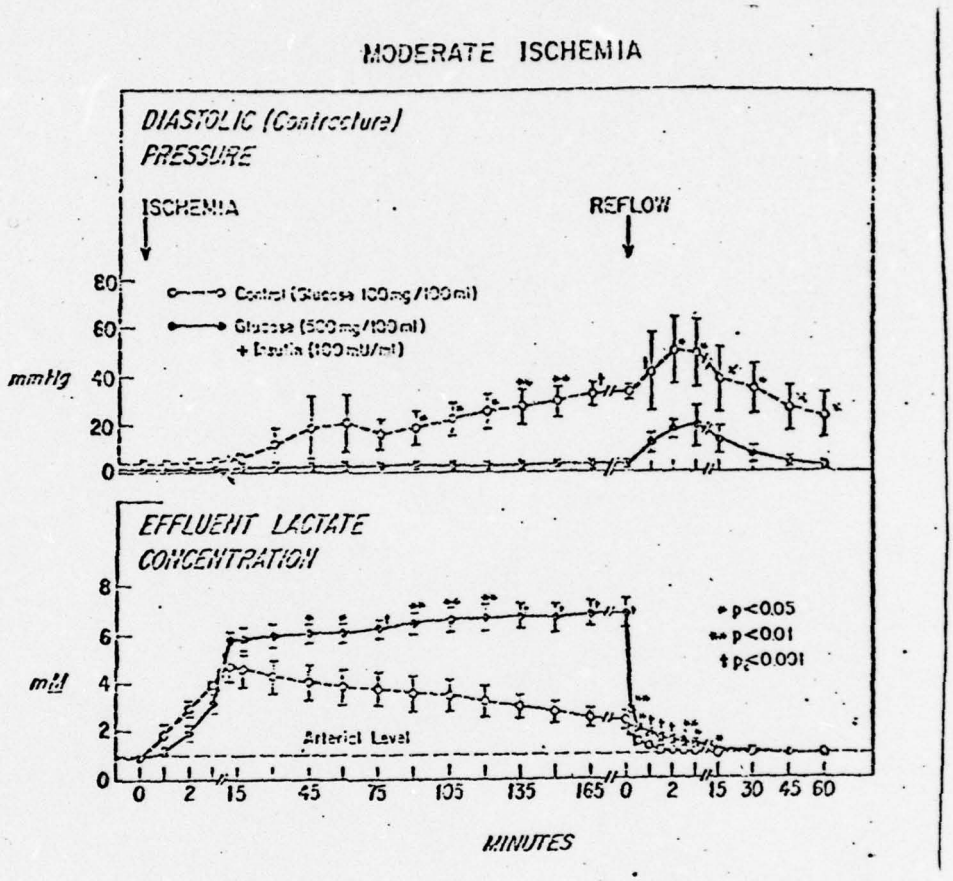


Figure 9. Effect of hyperglycemia and insulin on contracture and lactate production during moderate ischemia. (See text for discussion.)(n=25 in each group)

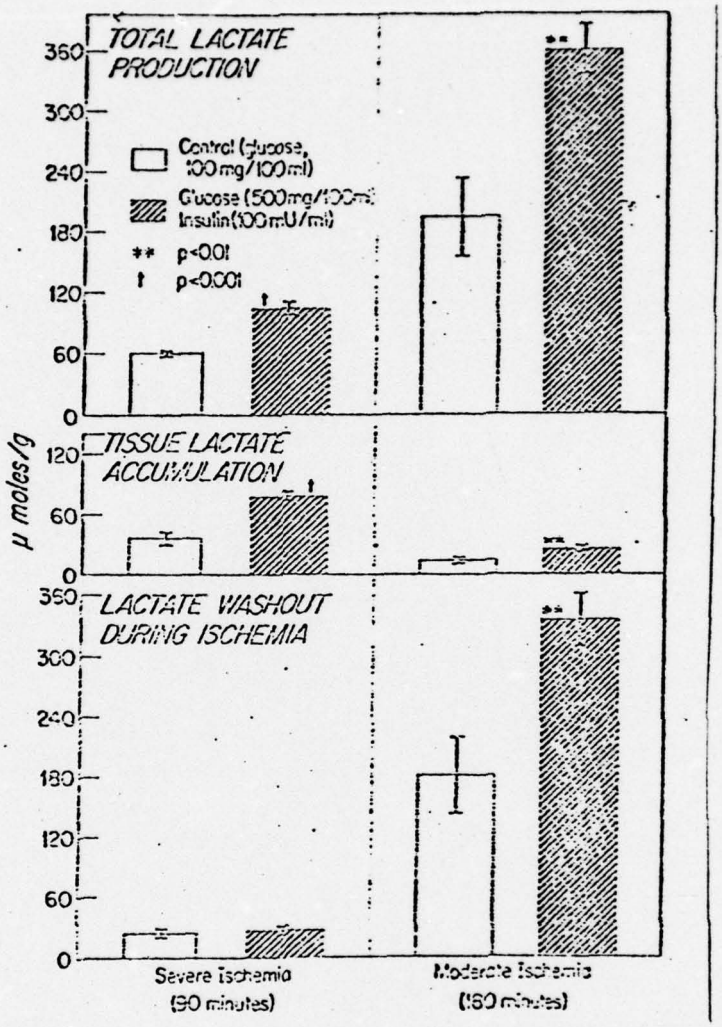


Figure 10. Effect of hyperglycemia and insulin on ischemic lactate metabolism. (See text for discussion.)

Table 2. Effect of Hyperglycemia and Insulin on Ischemic Rabbit Myocardial Tissue Levels of ATP

	5.5mM Glucose No Insulin	27.5mM Glucose & 100mU/ml Insulin	P
Moderate Ischemia (180 min)	5.3±0.7* (n=9)	7.8±0.5 (n=9)	<.05
Severe Ischemia (90 min)	4.1±0.2 (n=8)	6.1±1.0 (n=8)	0.05-0.10

* Tissue ATP in μmole/gm dry wt ± SEM at end of ischemic period.

Pre-perfusion myocardial ATP content was 14.3±1.2 μmole/gm dry weight (n=8). After 180 min of oxygenated perfusion with 100 mg% glucose as substrate it remained at 14.1±1.5 μmole/gm dry weight (p=N.S.). Thus, under oxygenated conditions with the isolated heart developing ventricular pressures in the range of 80-90 mm Hg at a rate of 180/min tissue ATP level is stable in this preparation.

insulin increased total ischemic lactate production from 60 to 100 μmoles/gm for the 90 min period. This increase in lactate production resulted in an increase in tissue lactate accumulation since little increase in lactate washout occurred during ischemia at this severely ischemic flow rate. In contrast, during moderate ischemia, the high glucose and insulin substrate also markedly stimulated total lactate production but the higher rate of ischemic tissue perfusion washed out the additional lactate which was produced, so that tissue accumulation of lactate was much less than during severe ischemia. Under conditions of moderate ischemia the high glucose and insulin substrate resulted in a significantly higher tissue ATP level at the end of the ischemic period; with severe ischemia tissue ATP levels were also increased in the high glucose and insulin group but the difference was of borderline statistical significance. Tissue ATP levels were preserved at a higher level with a combination of moderate ischemia, high glucose and insulin than severe ischemia high glucose and insulin.

In summary, these studies demonstrated a protective effect of hyperglycemia and insulin on the isolated ischemic myocardium. However, the ability of this high glucose and insulin substrate to protect ischemic myocardial tissue depended upon the severity of the ischemic condition and the duration of the ischemia. During the first 30 to 45 min the hyperglycemia - insulin substrate did not increase the rate of glycolysis as estimated from the effluent venous lactate concentration, suggesting that during the initial 30 to 45 min tissue glycogen stores provide adequate substrate for the glycolytic pathway; however, with longer periods of ischemia, as tissue glycogen levels diminish, the exogenous substrate becomes more critical. Thus, previous studies which failed to demonstrate a beneficial effect of hyperglycemia and insulin during myocardial ischemia may have failed to do so because of the relatively short protocols employed (generally less than 30 min of ischemia) or failure to impose the degree of ischemia for which hyperglycemia and insulin is beneficial. Failure to use an adequate sample size may also account for some previous negative studies by others.

Dissociation of Oxygen Consumption From Contractile Work After Ischemic Injury

We previously demonstrated that cardiac muscle was sensitive to relatively small changes in the severity of the ischemic condition. Severe ischemia for 30 minutes led to irreversible injury while moderate ischemia caused a reversible depression of function (Apstein et al, Graded Global Ischemia and Reperfusion; Cardiac Function and Lactate Metabolism. Circulation 55: 864-872, 1977.

The subcellular site of the difference between reversible and irreversible injury was studied by assessing the relationship between oxygen consumption and mechanical work and considering this relation to reflect mitochondrial integrity. The relationship between myocardial oxygen consumption and contractile work before and after severe ischemic injury in the rat heart is shown in Figure 11. Contractile work was determined by measuring the area under the ventricular developed pressure curve and is expressed in units of pressure seconds/min. The relationship between oxygen consumption and contractile work was constant during the control oxygenated period. With reperfusion after 30 minutes of severe ischemia, myocardial oxygen consumption occurred in excess of the amount of contractile work which was achieved. The same phenomenon was demonstrated in the post-ischemic rabbit heart (Figure 12). In the rabbit heart there was also a marked decrease in absolute (as well as relative) oxygen consumption, suggesting injury to respiratory enzymes in addition to uncoupling.

Although lactate accumulated in the myocardium in both the rat and rabbit during the period of ischemia, lactate washout was complete within 3 minutes of reperfusion. Myocardial oxygen consumption remained constant for 30 minutes of reperfusion, and therefore the increase in post-ischemic oxygen consumption cannot be attributed to a "lactate debt."

Thus after a period of severe ischemia both the rat and rabbit heart demonstrated a rate of myocardial oxygen consumption which was disproportionate to the resulting contractile function. Uncoupling of mitochondrial oxidative phosphorylation was thought to be the basis for the increased rate of oxygen consumption without translation of the oxidative process into mechanical work. The increased tissue utilization of oxygen, under conditions of limited oxygen delivery may be especially deleterious and may accelerate further ischemic injury.

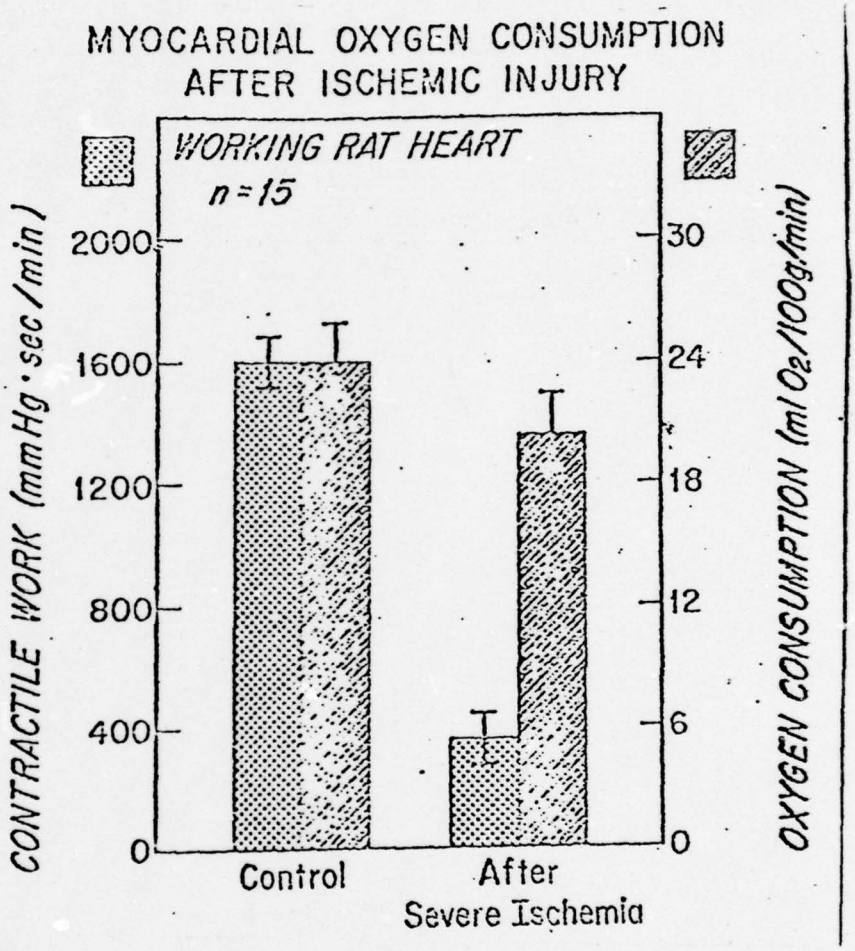


Figure 11. See text for discussion.

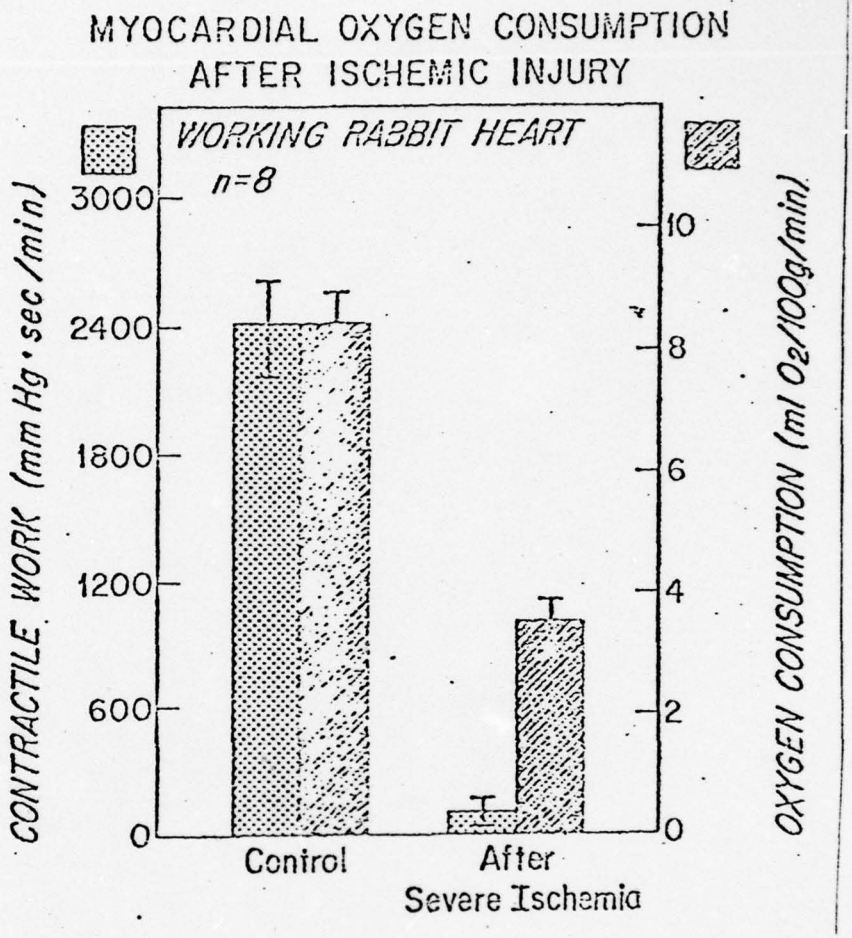


Figure 12. See text for discussion.

Blood Perfused Heart Experiments

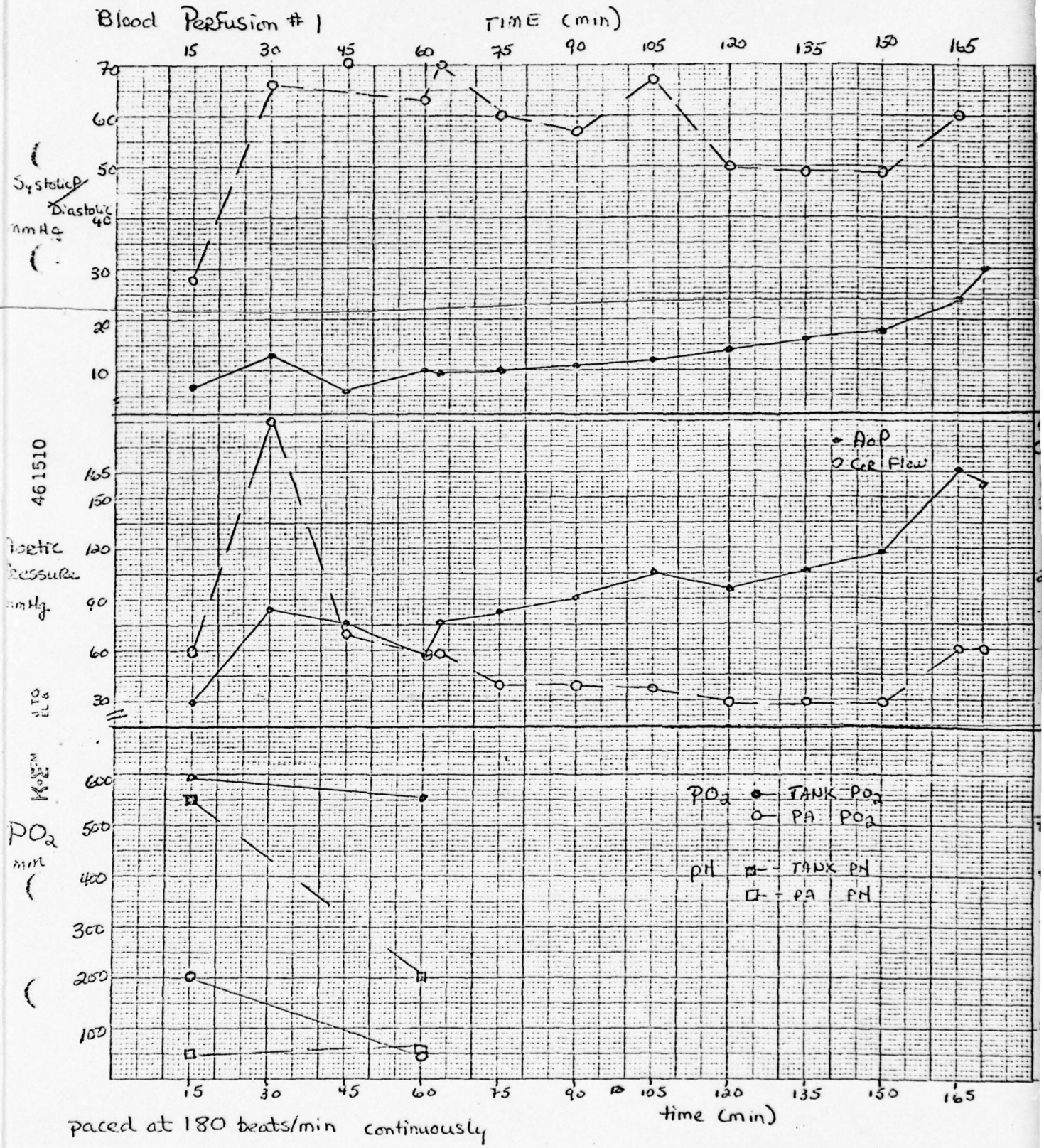
Utilizing the methodology described above we have completed a series of nine isolated blood perfused heart experiments using resuspended washed outdated human red blood cells without platelets. The results of each experiment are are shown in the following figures. In general, stable contractile performance was achieved for two hours with some hearts performing stably for a longer period of time. For example, heart #5 was stable for 165 min. Heart #7 showed a decrease in contractile function after 75 min, but remained at a stable level of contractility with minimal contracture for four hours. In some hearts, systolic and diastolic pressures were recorded while the heart was allowed to beat at its own spontaneous rate and also during pacing at 180 beats per minute so that the hearts could be compared at a common and constant heart rate.

The following figures show the parameters measured during the experiment. The upper panel shows the systolic and diastolic pressures. The difference between these two pressures represents the contractile effort of the heart as measured by the developed pressure which is generated by the left ventricle. An increase in the diastolic pressure represents constracture of the ventricle on the intraventricular balloon.

The aortic pressure and coronary flow are plotted on the same panel. Aortic pressure in our perfusion apparatus is equal to the pressure head which provides coronary perfusion pressure. Therefore, the relationship between the aortic pressure and coronary flow is a measure of coronary vascular resistance. A rise in aortic pressure and decrease in coronary flow indicates a rise in coronary vascular resistance. In general, the coronary resistance was noted to increase over the course of the perfusion experiment. In most experiments, the blood perfusate pO_2 and pH is also recorded. The "tank" pH or pO_2 refers to the values in the arterial blood before it passes through the heart; the "PA" (pulmonary artery), pH or pO_2 refers to values after the blood has passed through the heart. The individual experiments are discussed below.

Blood Perfused Heart #1

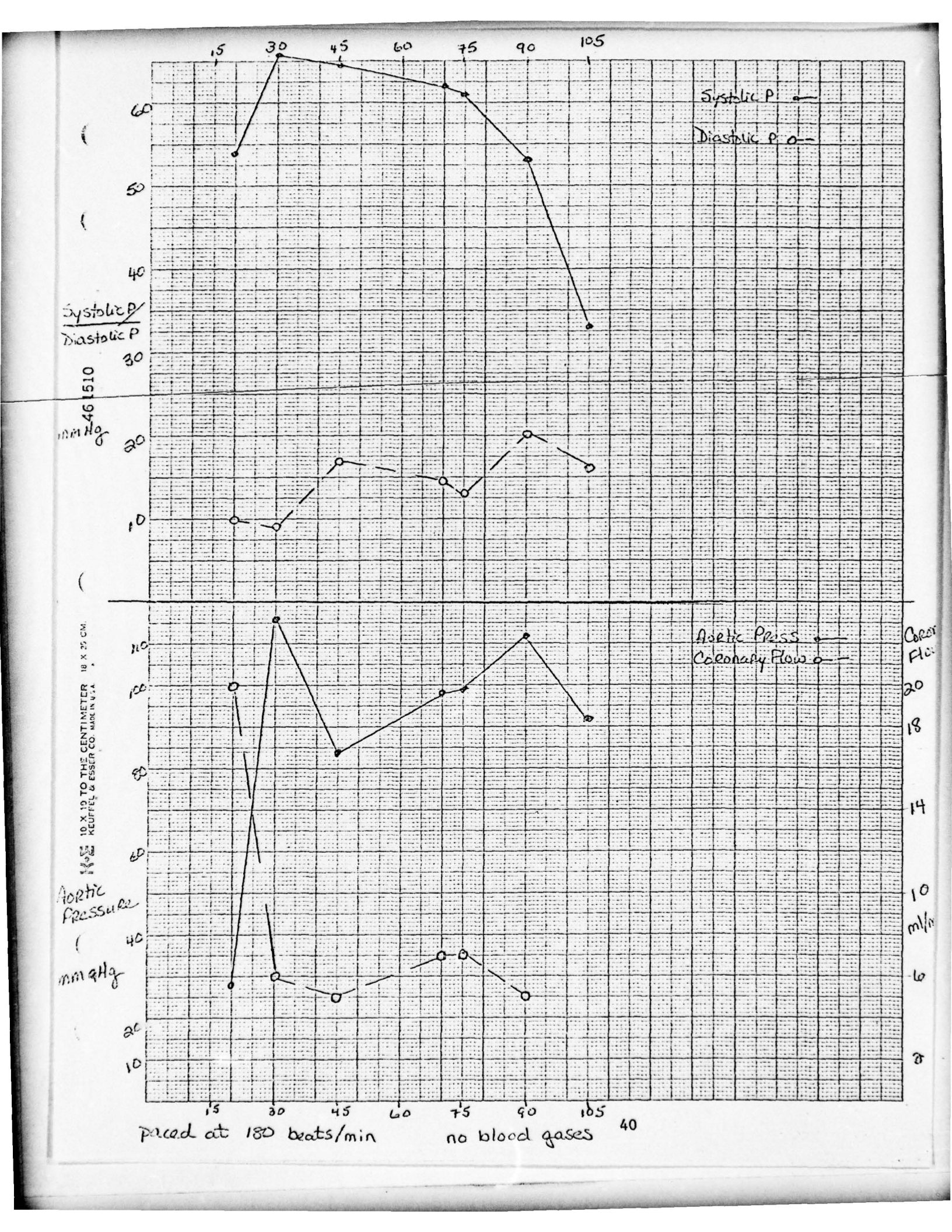
The hematocrit was 22, ionized calcium was 2.56 mEq/l. Developed pressure was stable for 105 min and then declined slightly as progressive contracture occurred starting at 105 min, and becoming more severe over the subsequent 45 min as the aortic pressure progressively increased concomitant with a decrease in coronary flow. The pH of the red blood cell suspension was 7.54 when it was first mixed, before equilibration with the 5% CO_2 gassing mixture. At the onset of the experiment the pH of the blood entering the heart was 7.36, the venous effluent was 7.27; after 60 min of perfusion the arterial pH fell to 7.30 without any apparent affect on contractility. In the first hour, the arterial pO_2 was in the 600 range and the coronary venous pO_2 was between 100 and 200. Subsequent blood gas measurements were not taken because of technical problems. It should also be noted in this experiment that the initial ionized calcium was 2.56 in the red blood cell suspension prior to gassing; repeated measurements after 60 min of perfusion were 2.20 and 2.68 respectively on duplicate samples.



paced at 180 beats/min continuously

Blood Perfused Heart #2

Initial pH of the red cell suspension was 7.74 after adjustment with sodium hydroxide. The Orion calcium electrode was not working and calcium chloride was added empirically. Subsequently, the Orion began to work but gave values on the same blood sample of 2.13 and 4.38 mEq/l. The hematocrit was 25. It was noted that the red blood cell suspension pH decreased from initial value of 7.74 to 7.36 progressively over half an hour; the reasons for this were unclear. After gassing with CO_2, the arterial pH was 7.16. pO_2 678 with a decrease in pO_2 to 222 after passage through the heart. This heart performed at a stable level for approximately 75 min and then had rapid contractile failure.


15
30
45
60
75
90
105
Systolic P
Diastolic P
60
50
40
30
20
10
Systolic P/
Diastolic P
mm Hg
Aortic Press
Coronary Flow
Aortic Pressure
mm Hg
Coron Flo
20
18
14
10
ml/m
6
8
110
100
80
60
40
20
10
10 X 10 TO THE CENTIMETER 18 X 25 CM.
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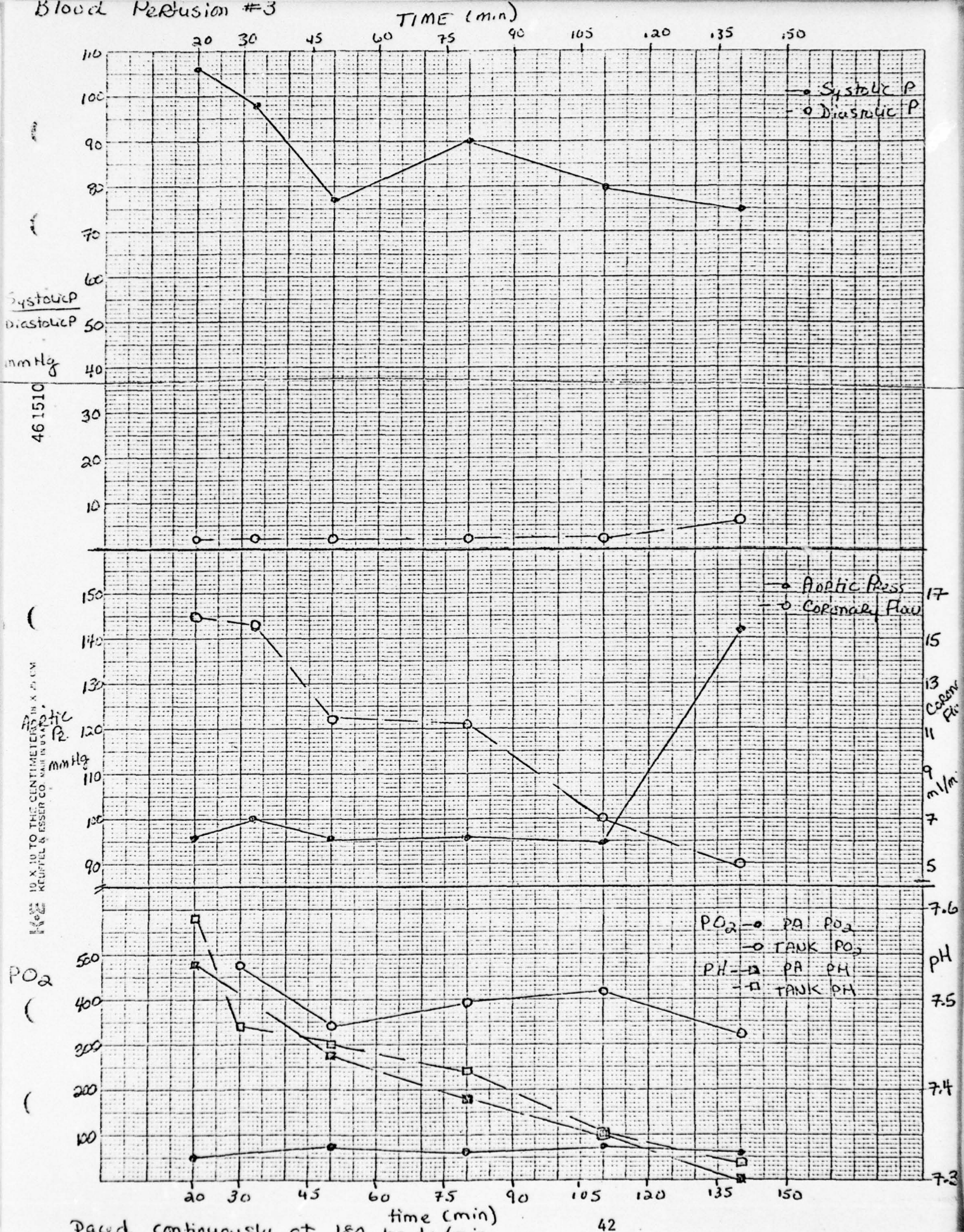
paced at 180 beats/min
no blood gases
40

Blood Perfused Heart #3

The hematocrit was 40%. The calcium electrode was non-functional and calcium was added to the perfusate empirically to bring it into an appropriate range based on the previous experiments. However, this could not be verified. Nonetheless, initial contractile function was excellent and leveled off to an acceptable level of 75 mm Hg developed pressure which lasted for about 140 min. Coronary vascular resistance progressively increased as indicated by the progressive decrease in coronary flow from an initial value of about 15 ml/min to 5 ml/min with aortic pressure being held constant at 95 mm Hg. In the last 15 min there was a marked increase in coronary vascular resistance as manifested by the increase in aortic (coronary perfusion) pressure and the continued decrease in coronary flow. The pH of the blood perfusate progressively decreased during the course of the experiment from the alkalotic to acidotic range. Oxygen extraction as measured by the arterial and venous pO_2's remained relatively constant despite the progressive decrease in coronary flow; the reasons for this are unclear and may simply reflect the lack of sensitivity of the pO2 measurement (as opposed to measuring oxygen content, which we do not have the capacity to do at the moment).

Hearts 1-3 were paced continuously at 180 beats per minute during the experiment.

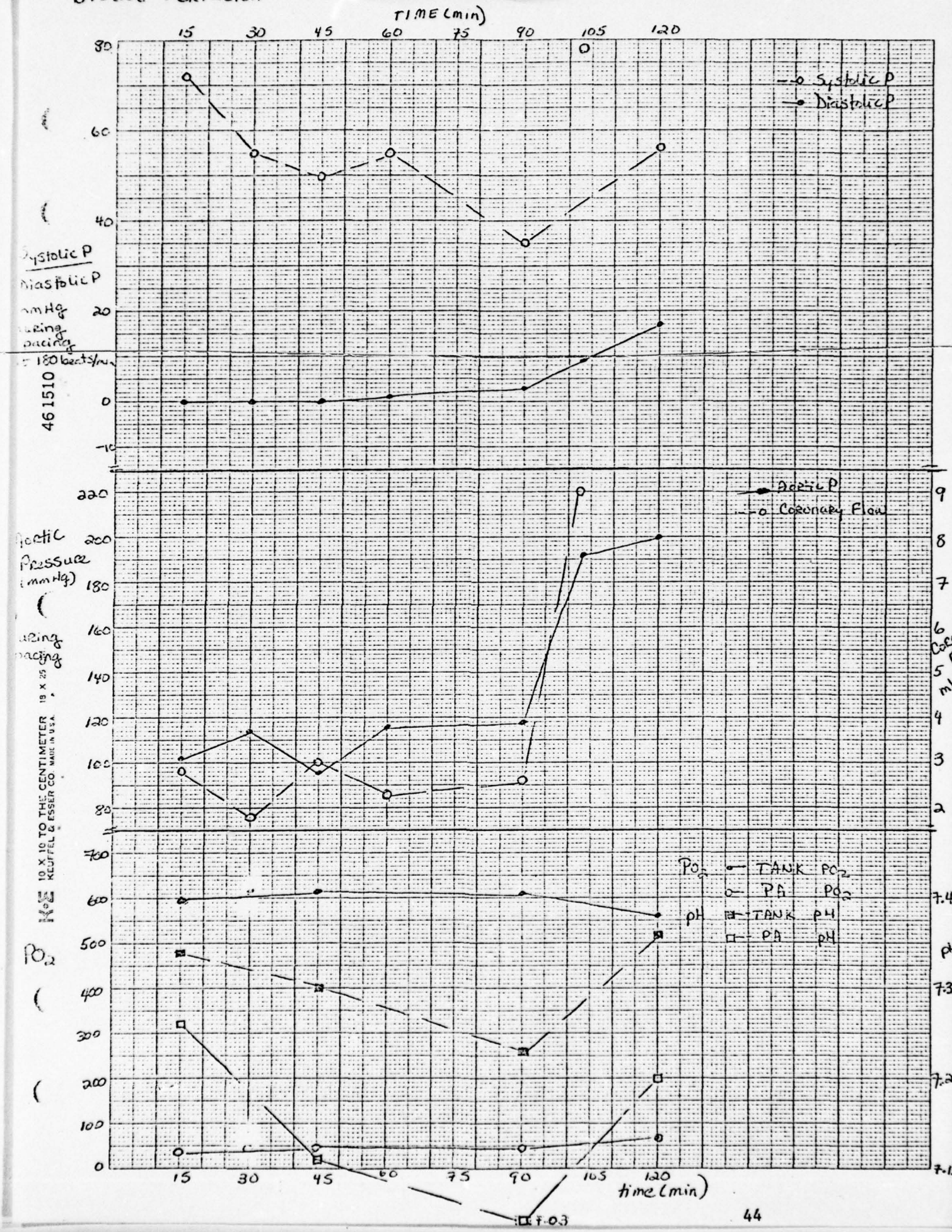
Blood Perfusion #3

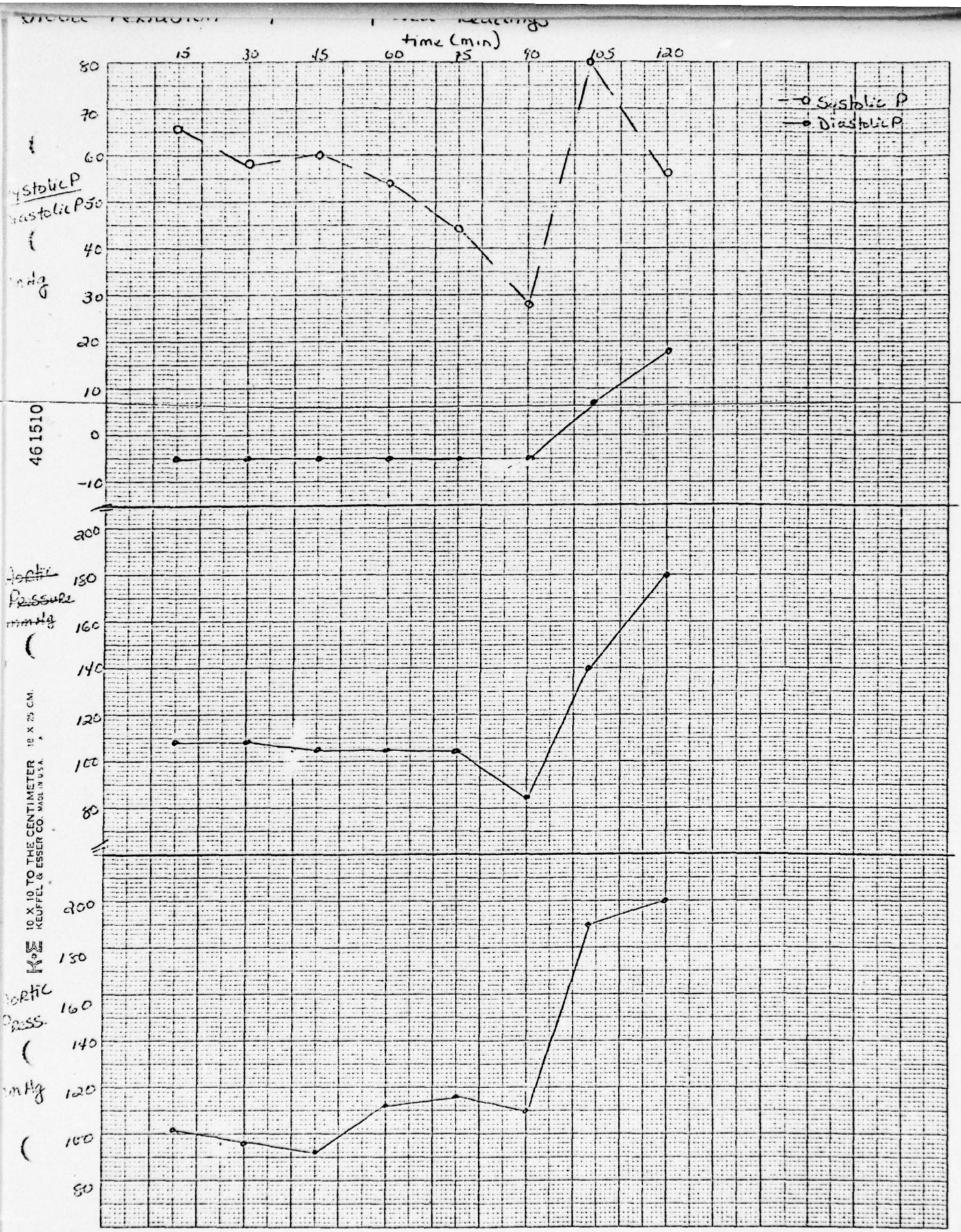


Paced continuously at 180 beats/min

Blood Perfused Heart #4

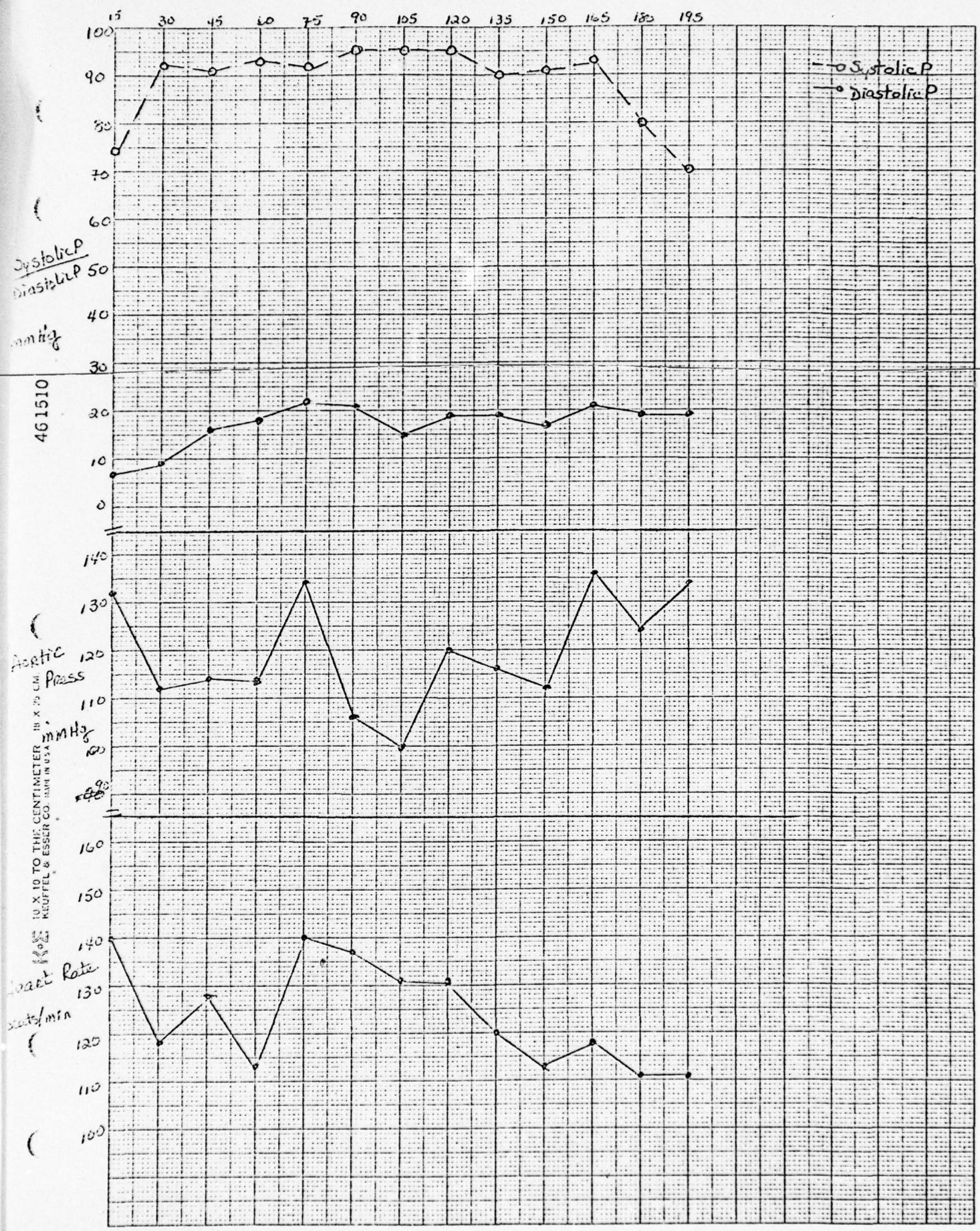
The hematocrit was adjusted to 31%. Once again the Orion calcium electrode was not operational and the calcium content was adjusted empirically. The heart had good initial contractile function which progressively decreased over 90 min, at which time significant contracture occurred. At the time of contractile failure the coronary flow was increased in an attempt to overcome the loss of contractility, but no significant improvement of contractility occurred, suggesting that the low level of function was not due to limitation of oxygen delivery. Oxygenation of the arterial blood and extraction by the myocardium was constant during the experiment. The pH of the perfusate blood progressively became acidotic except that in the last 30 min it spontaneously became more alkalotic. Beginning with blood perfused heart #4, pressures were recorded during both the unpaced and paced state; however, there was no significant difference in the overall performance of the heart regardless of whether pressure measurements were taken during the paced or unpaced state.


TIME (min)
15 30 45 60 75 90 105 120
Systolic P
Diastolic P
mmHg
Aortic Pressure (mmHg)
Coronary Flow
ml/min
P_{O_2}
pH
TANK P_{O_2}
PA P_{O_2}
TANK pH
PA pH
7.03
time (min)


time (min)
15
30
45
60
75
90
105
120
Systolic P
Diastolic P
80
70
60
50
40
30
20
10
0
-10
200
180
160
140
120
100
80
200
180
160
140
120
100
80
461510
10 X 10 TO THE CENTIMETER
KEUFFEL & ESSER CO. MADE IN U.S.A.

Blood Perfused Heart #5

The hematocrit was 31%. The ionized calcium level was adjusted by adding calcium as previously, however, the initial Orion reading was 1.46 mEq/l, a very low value. Based on this value, however, calcium was added to increase the ionized calcium to approximately 2.5 mEq/l; however, the next Orion reading was 5.5 mEq/l and the perfusion was carried out at this level of calcium (approximately twice normal). ~~Despite the high calcium~~ level, contractile function was good and stable for 165 min. The diastolic contracture pressure increased earlier than usual; this might have been due to the hypercalcemia. The heart rate was relatively stable between 110 and 140 for most of the experiment when the heart was unpaced. When pressure measurements were taken during the paced rate of 180 min, some impairment of diastolic relaxation was noted which led to an increased diastolic pressure being measured. Aortic pressure and coronary flow were relatively constant during the experiment. Myocardial oxygen extraction was relatively constant. There was a progressive decrease in the arterial pH for the first 150 min, at which time further sodium hydroxide was added to correct the pH and bring it back to the normal range; however, alteration of the pH did not affect the decline in contractile function.


15
30
45
60
75
90
105
120
135
150
165
180
195
100
90
80
70
60
50
40
30
20
10
0
Systolic P
Diastolic P
Systolic P
Diastolic P
mmHg
140
130
120
110
100
90
Aortic Pressure mmHg
160
150
140
130
120
110
100
Heart Rate
beats/min
461510
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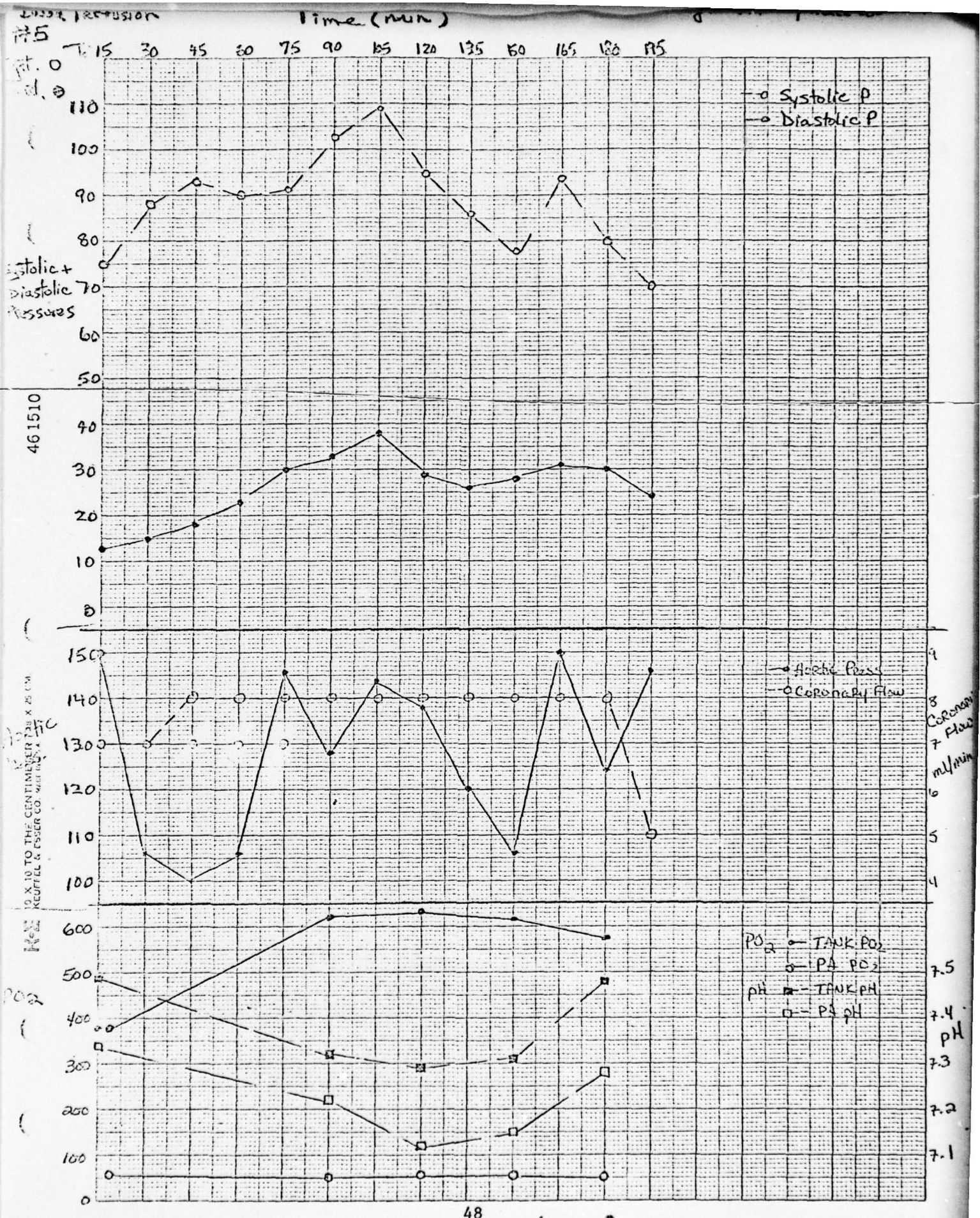
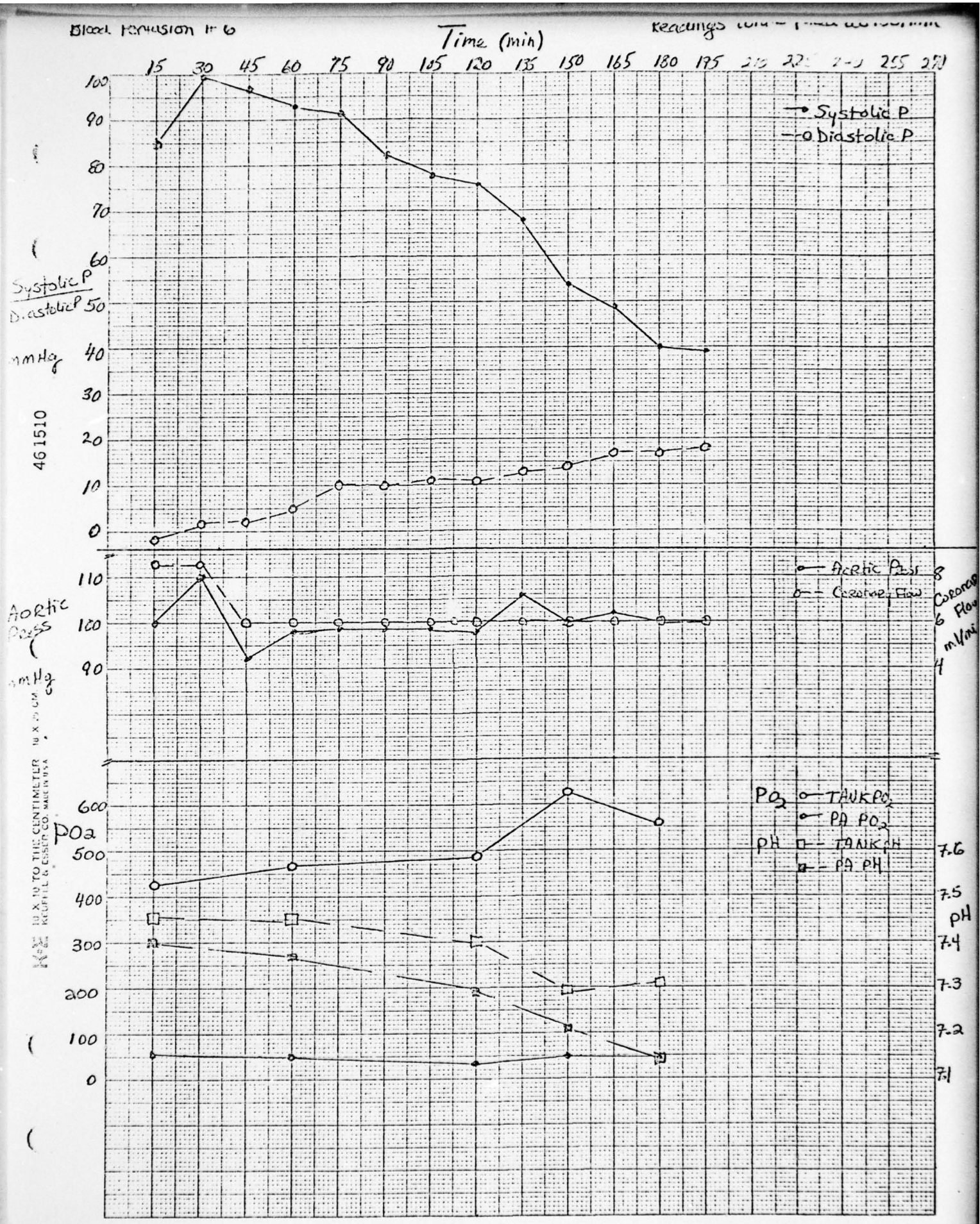

Time (min)
15 30 45 60 75 90 105 120 135 150 165 180 195
Systolic P
Diastolic P
Systolic + Diastolic Pressures
110 100 90 80 70 60 50
40 30 20 10 0
Aortic Press
Coronary Flow
Coronary Flow ml/min
150 140 130 120 110 100
9 8 7 6 5 4
PO2 — TANK PO2
PA PO2
pH — TANK pH
PA pH
600 500 400 300 200 100 0
7.5 7.4 7.3 7.2 7.1
pH

Blood Perfused Heart #6

The hematocrit was 26%. The calcium addition was less than in blood perfused heart #5 (which is hypercalcemic), but despite our calculations based on previous Orion measurements the final calcium concentration of the perfusate was 4.2 mEq/l, as measured by repeated Orion calcium electrode measurements at a pH of 7.4. Initial contractile function was good, but progressive contracture occurred starting at about 75 min and was associated with a progressive decrease in contractile function. Coronary flow and coronary vascular resistance were relatively constant throughout. Progressive acidosis of the circulating blood occurred, but oxygen extraction remained relatively constant. The same basic pattern held whether the measurements were taken when the heart was paced or unpaced.


Blood Perfusion # 6
Time (min)
15 30 45 60 75 90 105 120 135 150 165 180 195 255
Systolic P
Diastolic P
Systolic P / Diastolic P
mmHg
100 90 80 70 60 50 40 30 20 10 0
Aortic Press
Coronary Flow
mmHg
110 100 90
8 6 4
ml/min
PO2
600 500 400 300 200 100 0
PO2 TANK PO2
PA PO2
PH TANK PH
PA PH
7.6 7.5 7.4 7.3 7.2 7.1
pH
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10 X 10 TO THE CENTIMETER
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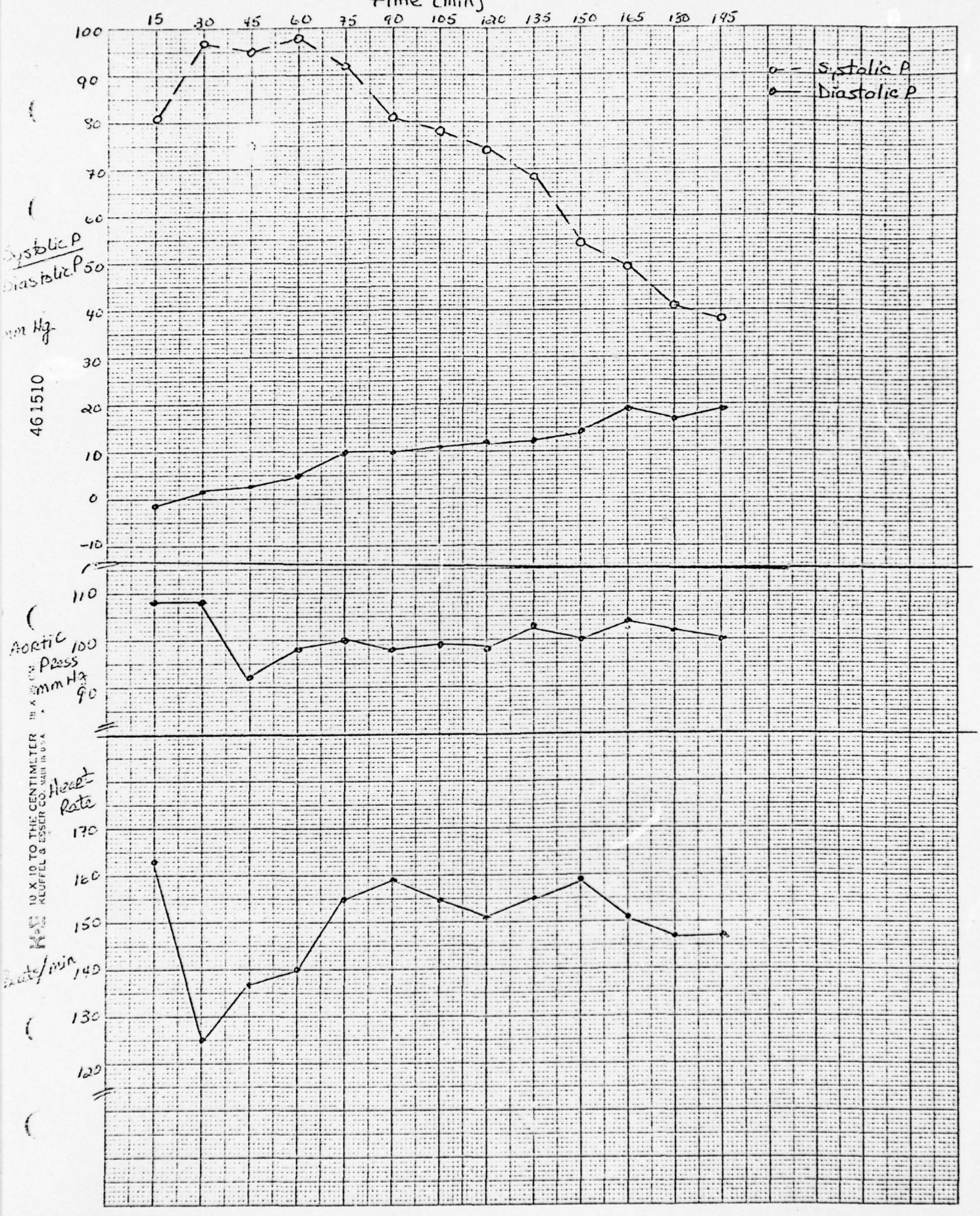
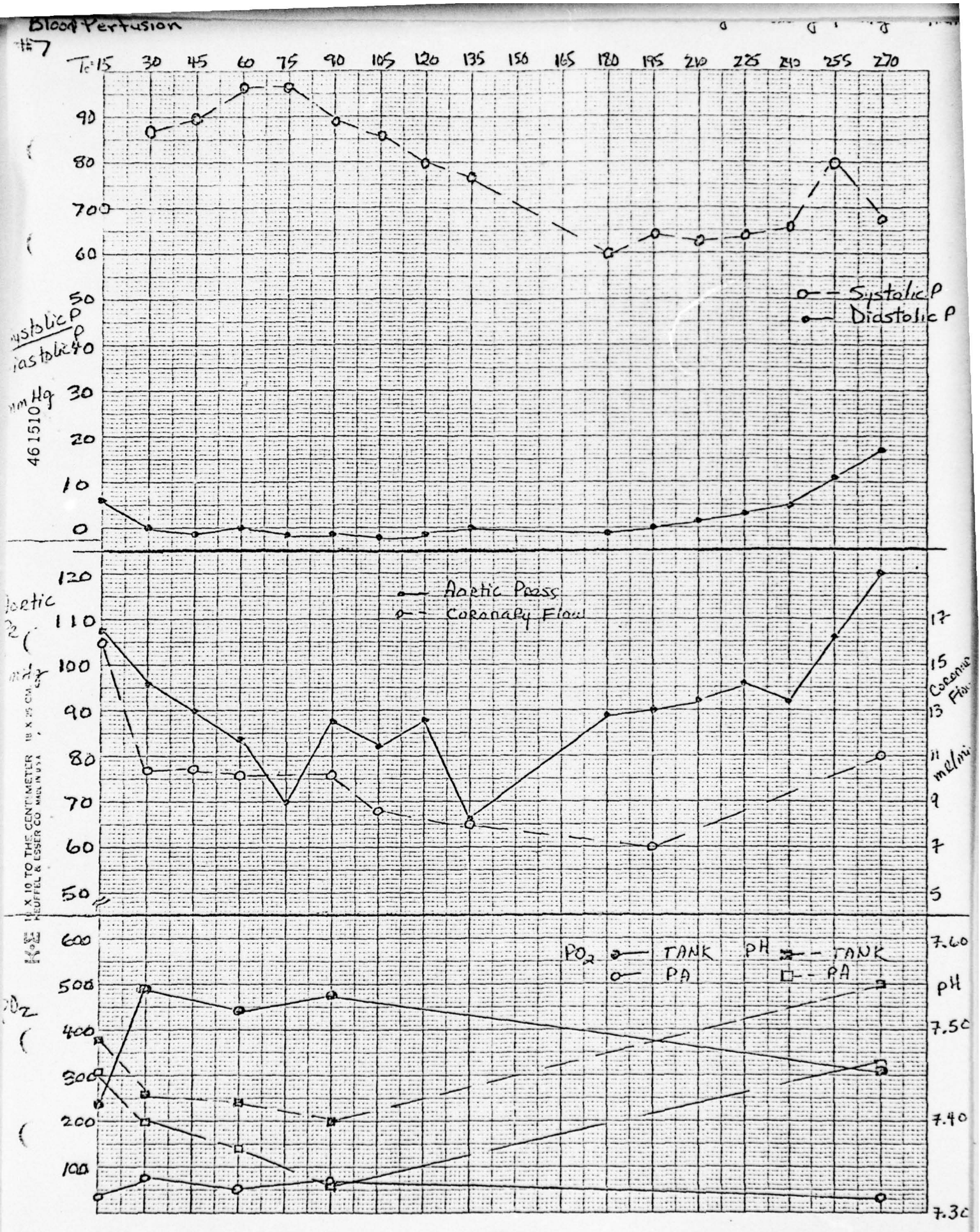
Blood Perfusion #6

unpaired Readings



Blood Perfused Heart #7

The hematocrit was 28%, the ionized calcium level was empirically adjusted within the normal range for this experiment and it remained between 2.73 and 2.78 mEq/l throughout the experiment. Contractile function was relatively stable for about 105 min and then contractility progressively decreased although significant contracture did not occur until approximately 240 min. There was a slight increase in coronary resistance. The arterial pH became slightly more acidotic during the experiment although oxygen extraction remained relatively constant. At 225 min the perfusate was switched to a freshly prepared RBC suspension, but no effect on contractile function was observed.


Blood Perfusion
#7
Tc 15 30 45 60 75 90 105 120 135 150 165 180 195 210 225 240 255 270
Systolic P
Diastolic P
mm Hg
Aortic Press
Coronary Flow
PO2 TANK
PA
pH TANK
PA
7.60
7.50
7.40
7.30
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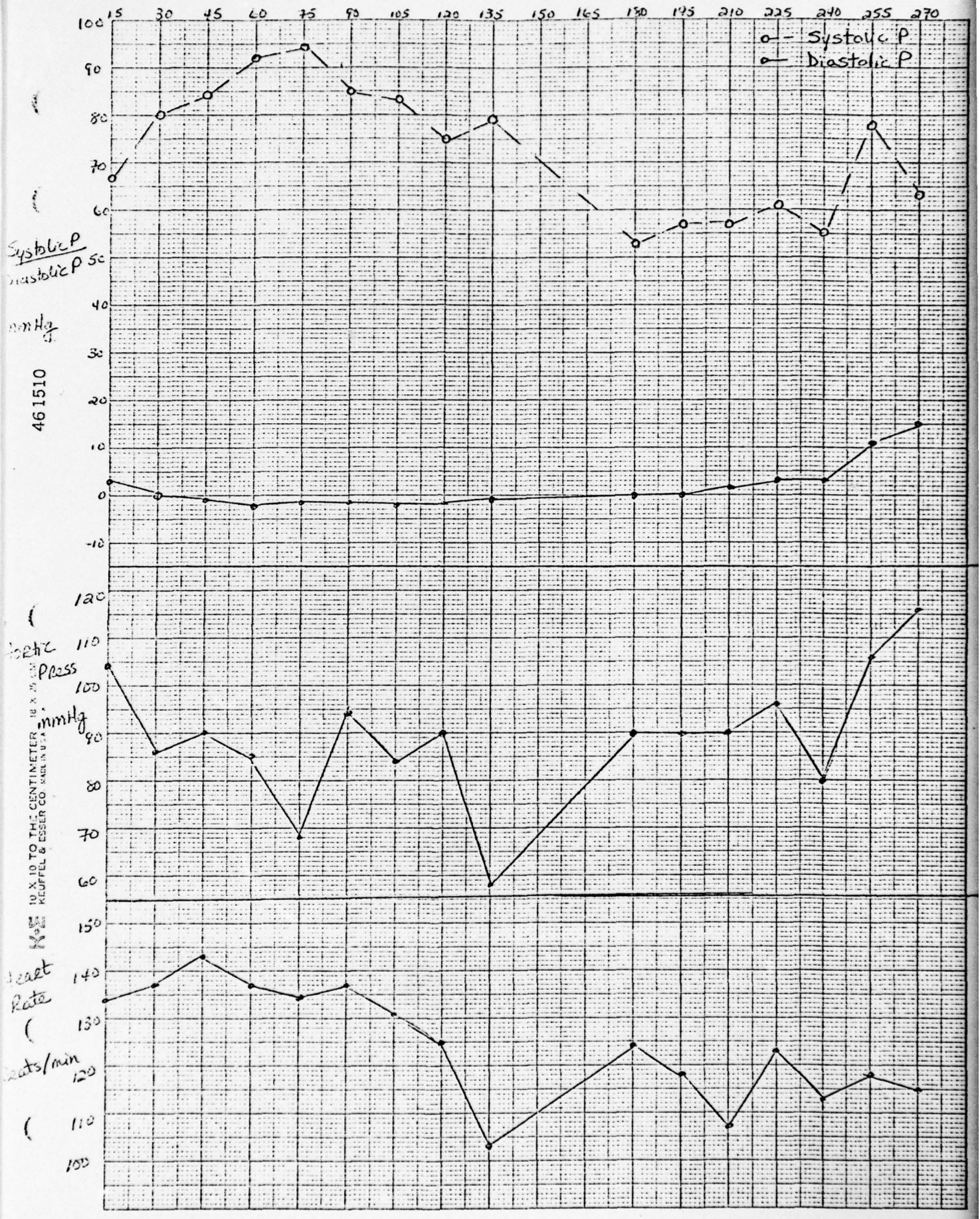
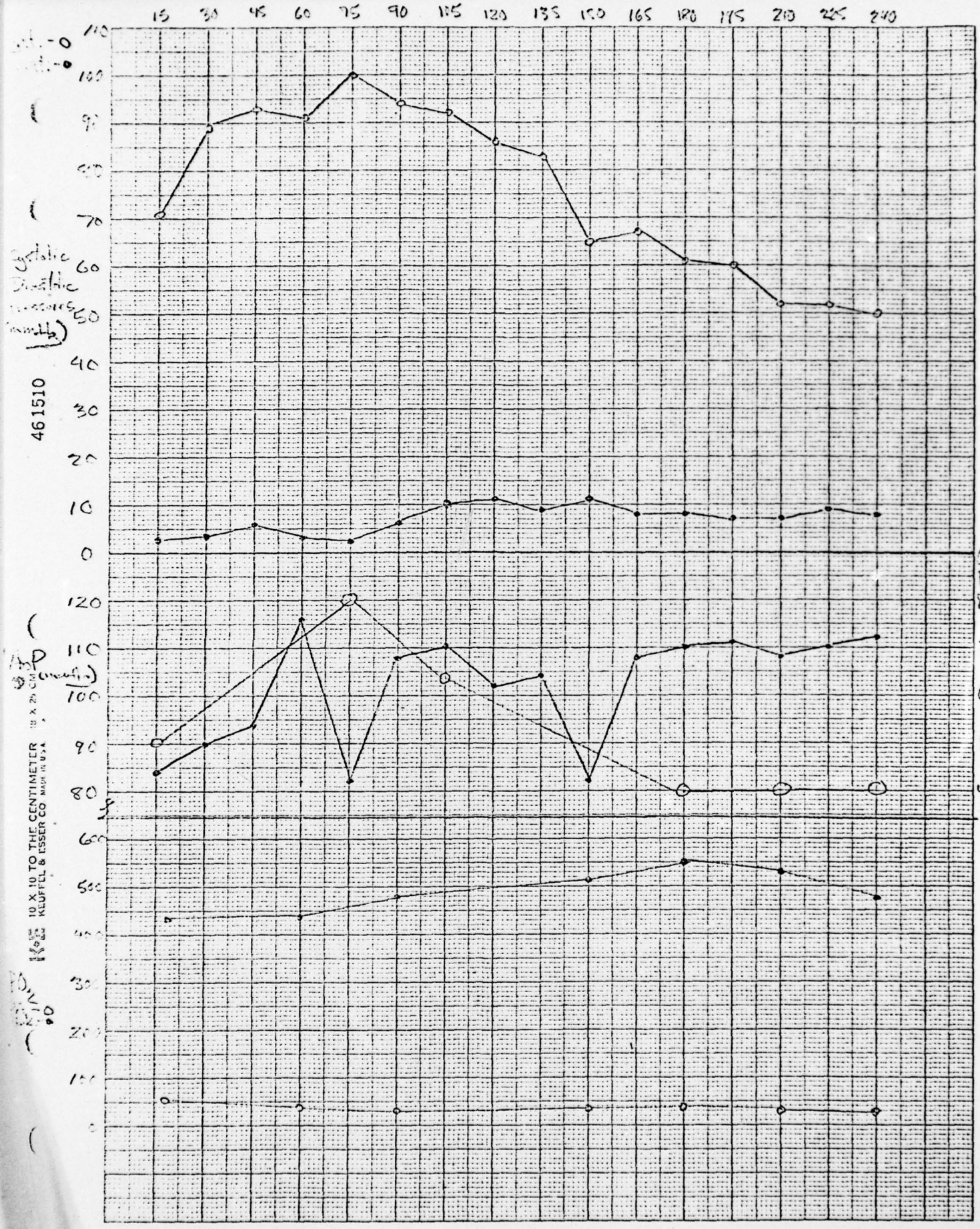
Blood Perfusion



Blood Perfused Heart #8

The hematocrit was adjusted to 30%. The ionized calcium level was 2.63 mEq/l initially, decreased to 2.44 at 150 min and 2.41 at 210 min. The arterial blood pH was initially alkalotic at 7.60 and decreased to 7.31 after 240 min of perfusion. Contractile function was initially good, but progressively decreased after approximately 105 min of perfusion time, although not much contracture occurred. Coronary flow progressively decreased after 70 min of perfusion time at relatively constant aortic pressure head, indicating that coronary resistance increased. Myocardial oxygen extraction remained relatively constant.


15 30 45 60 75 90 105 120 135 150 165 180 195 210 225 240
Systolic
Diastolic
461510
10 X 10 TO THE CENTIMETER
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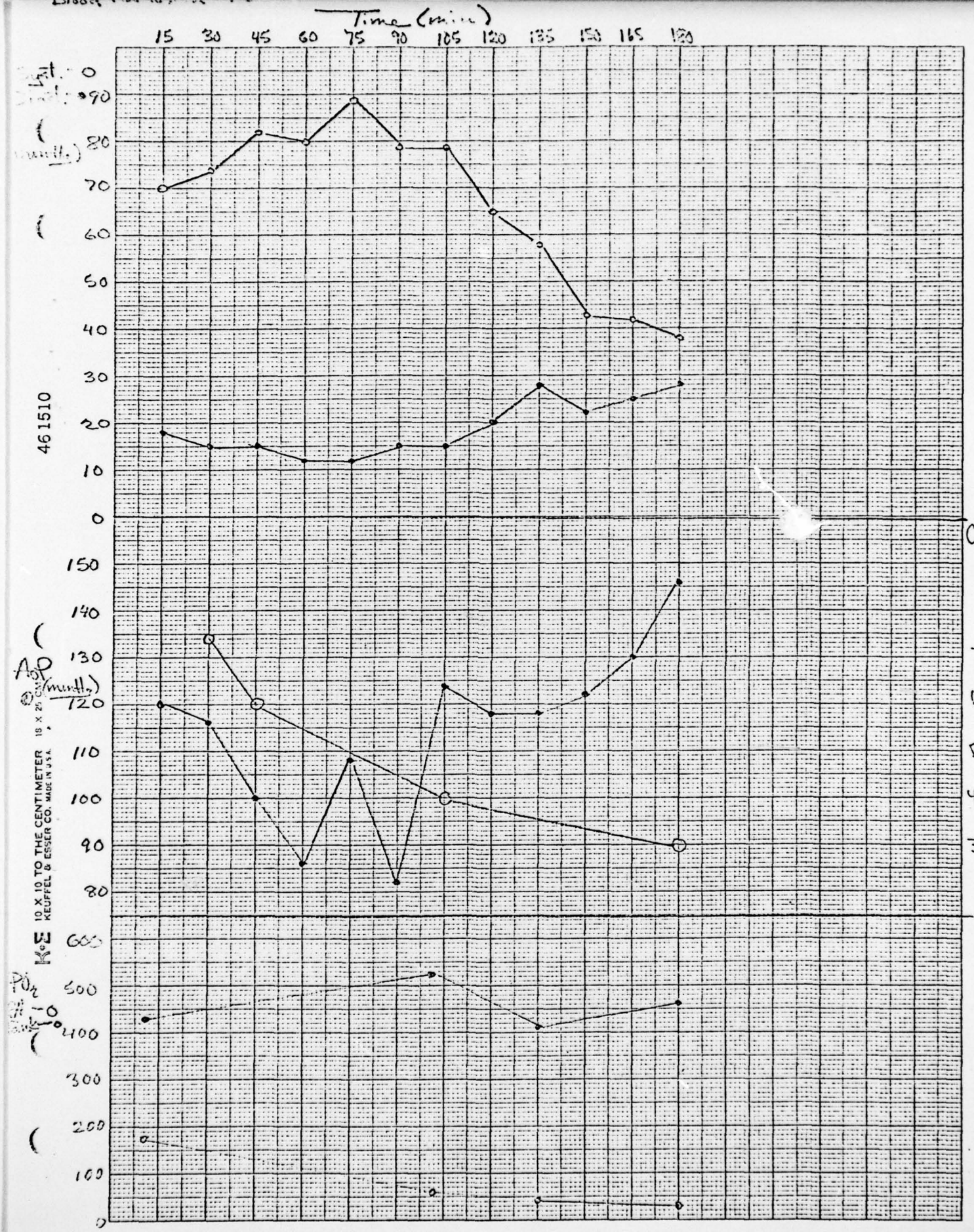
Blood Perfused Heart #10

(Blood perfused heart #9 was subjected to severe ischemia and reflow, and was not part of this series.)

Blood perfused heart #10 had a hematocrit of 27% and initial ionized calcium level of 2.57 mEq/1 at 10 min, 2.54 at 60 min. The initial arterial blood pH was 7.49 and decreased to 7.31 at 100 min, 7.22 at 135 min and 7.13 at 108 min (these values are on arterial blood after passing through the lung oxygenator). Contractile function was stable for 105 min after which contractility progressively decreased. Coronary resistance progressively increased as indicated by the decreasing flow rate, despite the high coronary perfusion pressure. Oxygen extraction remained relatively constant.


Time (min)
15 30 45 60 75 90 105 120 135 150 165 180
0 10 20 30 40 50 60 70 80 90
(mm Hg)
46 1510
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150
(mm Hg)
0 100 200 300 400 500 600
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Metabolic and electron microscopic evaluation of the blood perfused hearts is currently being done; these studies are in an incomplete form.

Discussion

In year one of this contract we have used our experimental preparation of the isolated heart to study the experimental shock state, modelled by a condition of reduced coronary arterial flow (ischemia), as would occur in the setting of arterial hypotension. The results show that this experimental preparation is ideally suited to these studies and provides an experimental basis for investigating interventions which could protect the heart during simulated shock.

As indicated in the Results section the methodology to evaluate tissue damage by measuring parameters of contractile function, diastolic compliance, coronary vascular resistance, tissue ATP levels, myocardial oxygen consumption, lactate production, and tissue ultrastructure have been developed and validated.

Our initial studies were directed toward evaluating the possible effect of selected plasma constituents on heart muscles subjected to the simulated shock state. Our results clearly demonstrate that a hyperglycemic plasma which contains insulin is protective to heart muscle subjected to moderate ischemia; the protective effect on cardiac muscle subjected to very severe ischemia is less clearcut. Since this level of hyperglycemia and insulinemia could be tolerated without adverse effects, such an intervention has potential applicability in the treatment of shock and trauma in a wide variety of settings. Accordingly, our results provide impetus for further study of this intervention in whole animals subjected to experimental shock.

It is of interest that the elevated glucose and insulin levels produced an increased amount of lactic acid production, but the increase in lactic acidosis did not appear to be detrimental to cardiac function. If such an intervention were applied to the whole animal the increase in lactic acidosis might need compensation by simultaneous administration of an alkalizing agent such as sodium bicarbonate.

The detailed results of our initial experiments in an isolated blood perfused heart preparation are presented. Stable performance has been achieved for approximately two hours in this series of hearts; then progressive contracture, a decline in cardiac function, and an

increase in coronary vascular resistance occurred. The suspended red cells also appear to be unstable and progressively become more acidotic. The reasons for the progressive acidosis of the red cell suspensions, and the progressive decline in cardiac function are not clear at this time, but these problems will be the subject of our next set of experiments.

Preliminary histological and ultrastructural inspection of our isolated hearts has shown a high degree of endothelial damage and resultant intercellular edema. Endothelial damage at the arterial or capillary level could also account for the increase in coronary vascular resistance. Accordingly, our immediate hypothesis is that the lack of stability of the preparation beyond two hours is due to endothelial damage; this damage may be decreased by adding platelets to the red cell suspension. Accordingly, in our next series of experiments, we plan to perfuse the isolated hearts with a red blood cell-platelet mixture.

It is also possible that the colloid content of our red cell suspension, as we now prepare it, is inadequate. The circulating albumin level of 2.5 grams % is roughly 50% of the normal value; however, we have been reluctant to increase this level because of the expense of the albumin, and because clinically, many patients walk around with a circulating albumin level of 2.5 gram % without any apparent cardiac dysfunction. However, should the addition of platelets not significantly improve performance, then a trial of increased colloid content would appear warranted.

Finally, we plan to alter the hemoglobin type of the red blood cells in order to test the effect of an altered oxyhemoglobin affinity state as outlined in the original contract proposal.

As indicated in the detailed results of the blood perfused hearts, great experimental difficulty has been encountered in adjusting the ionized calcium concentration of the red blood cell perfusate. This is a critical parameter since contractility is directly proportional to the ionized calcium level. Furthermore, an abnormally high or low ionized calcium level may contribute to premature deterioration of the isolated heart system. Other circulating electrolytes are probably also important in maintaining the

stability of the isolated heart preparation, but we currently do not have the capability of measuring these electrolyte levels. An improved methodology for monitoring the ionized calcium and other electrolyte levels appears to await better instrumentation.

Conclusions and Significant Accomplishments

1. An experimental isolated heart preparation has been developed to simulate the shock and trauma state and its characteristics have been defined in terms of contractile function, metabolism, and ultrastructure. Methodology has been developed to assess whether a given intervention is beneficial or deleterious to heart muscle subjected to experimental shock (ischemia).

2. Alteration of the plasma glucose and insulin level was demonstrated to be of significant benefit in protecting the heart muscle from experimental ischemia (shock). The beneficial effects of the elevated glucose and insulin consisted of improved contractility in the post-shock state, prevention of contracture during shock (ischemia) and maintenance of a higher tissue ATP level and greater glycolytic flux.

3. An isolate blood perfused heart preparation has been developed which is stable and functions in the normal range for approximately two hours. The groundwork has thus been developed for improving the stability of this isolated heart preparation to test an altered oxyhemoglobin state and assess the effect of red blood cells with normal, decreased or increased affinity for oxygen on normal or ischemic cardiac function.

8. Bibliography

Papers in preparation:

1. Protection of ischemic heart muscle with hyperglycemia and insulin.

2. Relationship between degree of ischemia and myocardial lactate production.

Abstracts

1. Apstein, C.S., Gravino, F.N., and Hood, W.B., Jr. Lactate concentration gradient is a poor quantiative index of myocardial ischemia. (Submitted to Annual Scientific Sessions of American Heart Association.)

2. Gravino, F.N., Frazer, J.C., and Apstein, C.S. Duration or severity of ischemia determine cardiac response to hyperglycemia + insulin. (Submitted to Annual Scientific Sessions of American Heart Association.)

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Heart, Cardiac, Myocardium, Shock, Trauma, Red Blood Cells, Ischemia, Glucose, Insulin.

20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

An experimental model of shock and trauma was developed in isolated rabbit hearts subjected to low levels of coronary arterial flow (ischemia). A high level of glucose and insulin was beneficial to the heart subjected to ischemia; equiosmolar mannitol had no effect. A blood-perfused rabbit heart was developed using outdated human red blood cells. The performance data of the blood-perfused heart under well-oxygenated conditions is reported.

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